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ORIGINAL ARTICLE

Assessment of Molecular Mechanisms and Potential Biomarkers in Bladder Urothelial Carcinoma

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ABSTRACT

Objective: Bladder cancer ranks 10th among the most common cancers worldwide, effecting mostly man than women. The aim of this study is to perform a detailed gene expression analysis of bladder urothelial carcinoma to reveal altered molecular mechanisms and to find potential biomarkers for this cancer.

Materials and Methods: Bladder urothelial carcinoma RNA-seq data from TCGA with normal bladder samples from GTEx were analyzed by using GEPIA. Differentially expressed genes were annotated to GO-BP and KEGG pathway terms with DAVID and PPI networks were constructed by STRING. The association of upregulated cell cycle pathway proteins and patient survival was further investigated.

Results: Upregulated genes mainly annotated to cell cycle, p53 signaling and oocyte meiosis and maturation pathways and cell cycle related GO-BP terms. Downregulated genes mostly annotated to adhesion, ECM-receptor interaction, vascular smooth muscle contraction and cardiomyopathy related KEGG pathways and muscle related GO-BP terms. The protein products of six cell cycle genes, which were upregulated in bladder urothelial carcinoma, showed significant association with patient survival.

Conclusion: The results of this study showed altered molecular mechanisms and increased our understanding of bladder urothelial carcinoma, proposed potential prognostic biomarkers.

Keywords: Prediction, protein-protein interaction networks, pathway analysis, gene expression, patient survival

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INTRODUCTION

Bladder cancer ranks 10th among the most common cancer forms worldwide, with an anticipated 549,000 new cases and 200,000 deaths in 2018 [1]. Bladder cancer is more prevalent in man than women. It is the 6th most common cancer and ranks 9th in cancer death causes in man [1]. Bladder cancer has adaptable metastatic potential and bones, liver, peritoneum, lung and lymph nodes are the common metastasis sites of bladder cancer [2]. Genetic predisposition has been proposed to have a significant effect on bladder cancer incidence [3]. Genetic mutations of several cell division and cell cycle genes, such as HRAS, TP53, CCND1 and RB1, have been associated with bladder cancer [4]. Although exploring underlying genetic mutations are important in bladder cancer, it is also essential to reveal gene expression alterations and molecular mechanisms, especially proliferation, metastasis and cell cycle mechanisms, for facilitating diagnosis and treatment approaches in bladder cancer.

Re-analysis of gene expression datasets is a powerful tool to search altered mechanisms of cancer. Previously, studies were performed with re-analysis of microarray datasets from GEO database of

tumor and control samples to reveal altered gene expression mechanisms of bladder urothelial carcinoma [5, 6]. The increased expression of genes in bladder cancer found to be annotated to negative regulation apoptotic process, salmonella infection, amoebiasis, malaria pathways and rheumatoid arthritis pathways, while downregulated genes were annotated to oxidative phosphorylation, taurine and hypotaurine metabolism, vitamin digestion and absorption, arthritis and vibrio cholerae infection pathways [7]. Enrichment of upregulated genes in cell division and protein binding, enrichment of downregulated genes in extracellular matrix organization and complement and coagulation cascades was also reported [5].

Studies with TCGA datasets of bladder cancer mainly focus on genomic analysis of DNA alterations, including variations and mutations, and related pathways [8-11]. According to DNA alteration data, several critical pathways, including cell cycle pathway, have been shown to be dysregulated in bladder cancer [8, 10]. At the gene expression level TCGA datasets were mainly used to identify molecular subtypes of bladder cancer or finding important miRNA or lncRNA signatures [8, 10, 12, 13]. Therefore, studies using TCGA RNA-seq data of bladder cancer to search for significant mRNA expression alterations and molecular mechanisms will contribute to bladder cancer research.

This study presents a detailed RNA-seq gene expression analysis with high number of bladder urothelial carcinoma and normal samples. Gene ontology (GO) terms for biological process (BP), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses and protein-protein interaction network constructions were performed. The effect of gene expression alterations of selected gene products on patient survival was also investigated.

MATERIALS and METHODS

RNA-seq Analysis of Bladder Urothelial Carcinoma

Bladder urothelial carcinoma RNA-seq data included 404 tumor samples and 19 normal samples from TCGA (The Cancer Genome Atlas) and 9 normal bladder samples from GTEx (Genotype-Tissue Expression). RNA-seq data was analyzed by using GEPIA (http://gepia.cancer-pku.cn/, [14]). ANOVA was applied to identify differentially expressed genes between tumor and normal samples. The Benjamini and Hochberg false discovery rate method was applied for adjusting p-value to calculate multiple testing adjusted q-value. |Log2FC|=2 and q-value=0.001 were designated as cutoffs to obtain differentially expressed genes.

GO-BP and KEGG Pathway Analyses of Differentially Expressed Genes

GO-BP and KEGG pathway analyses were performed by using Database for Annotation Visualization and Integrated Discovery version 6.7 (DAVID; https://david.ncifcrf.gov/, [15, 16]). Benjamini-Hochberg corrected p-value <0.05 was considered as significant.

Construction of Protein-Protein Interaction (PPI) Network

STRING version 11.0 (http://string-db.org/, [17]) was used to evaluate PPI information. Differentially expressed upregulated and downregulated genes were loaded to STRING, respectively. For network construction minimum required interaction score was set to highest confidence: 0.900.

Survival Analysis

The cancer patient survival analysis for selected genes was evaluated with Kaplan–Meier plotter tool (http://kmplot.com/analysis/index.php, [18]). Patient cohorts, divided in two groups according to quantile expressions, were compared and hazard ratio with 95% confidence intervals and log rank p-value were calculated and provided with a survival curve for overall survival. The log rank p-value<0.05 considered as significant.

RESULTS

Differentially Expressed Genes for BLCA

RNA-seq expression data for bladder urothelial carcinoma (n=404) was compared with related normal samples (n=28) to obtain differentially expressed genes. According to this comparison, 730 genes were found to be differentially expressed between tumor and normal samples. Of the 730 genes, 171 genes were found to be upregulated, while 559 genes were downregulated.

Functional Annotation of Differentially Expressed Genes

To identify molecular mechanisms that were altered in bladder urothelial carcinoma, upregulated and downregulated differentially expressed genes were annotated to GO-BP terms and KEGG pathway terms. Upregulated genes mainly annotated to cell

Table 1. The KEGG pathway annotations and top 20 GO-BP terms of differentially expressed upregulated genes in bladder urothelial carcinoma (Benjamini<0.05).

KEGG pathway term	Count	P-value	Benjamini
hsa04110:Cell cycle	18	1.34E-16	4.88E-15
hsa04114:Oocyte meiosis	11	3.16E-08	6.95E-07
hsa04914:Progesterone-mediated oocyte maturation	8	9.71E-06	1.42E-04
hsa04115:p53 signaling pathway	6	3.30E-04	3.63E-03
GO-BP term	Count	P-value	Benjamini
GO:0022403~cell cycle phase	60	4.47E-59	3.94E-56
GO:0000279~M phase	56	1.27E-58	5.58E-56
GO:0007049~cell cycle	68	2.02E-53	5.93E-51
GO:0022402~cell cycle process	61	2.64E-52	5.82E-50
GO:0000278~mitotic cell cycle	53	5.47E-51	9.64E-49
GO:0000280~nuclear division	45	1.06E-49	1.55E-47
GO:0007067~mitosis	45	1.06E-49	1.55E-47
GO:0000087~M phase of mitotic cell cycle	45	2.50E-49	3.14E-47
GO:0048285~organelle fission	45	7.14E-49	7.86E-47
GO:0051301~cell division	41	7.98E-38	7.81E-36
GO:0007059~chromosome segregation	20	9.32E-23	8.21E-21
GO:0007017~microtubule-based process	26	5.13E-20	4.11E-18
GO:0007051~spindle organization	15	6.01E-19	4.41E-17
GO:0051726~regulation of cell cycle	27	2.71E-18	1.84E-16
GO:0000226~microtubule cytoskeleton organization Organization	19	2.85E-16	2.10E-14
GO:0007346~regulation of mitotic cell cycle	19	5.20E-16	3.26E-14
GO:0000070~mitotic sister chromatid segregation Segregation	11	2.80E-13	1.54E-11
GO:0000819~sister chromatid segregation	11	3.80E-13	1.97E-11
GO:0010564~regulation of cell cycle process	14	1.28E-11	6.27E-10
GO:0000075~cell cycle checkpoint	13	1.43E-11	6.61E-10

KEGG, Kyoto Encyclopedia of Genes and Genomes; GO-BP, Gene Ontology-Biological Process; Count, no. of genes in term; p-value, modified Fisher exact p-value, EASE score; Benjamini, Benjamini-Hochberg corrected p-value.

Downregulated genes in bladder urothelial carcinoma cells mostly annotated to adhesion, ECM-receptor interaction, vascular smooth muscle contraction and cardiomyopathy related KEGG pathways (Table 2), muscle related GO-BP terms, such as muscle organ development, construction and system process, biological adhesion, and circulatory system related GO-BP terms (Table 2).

Table 2. The KEGG pathway annotations and top 20 GO-BP terms of differentially expressed downregulated genes in bladder urothelial carcinoma (Benjamini<0.05).

KEGG pathway term	Count	P-value	Benjamini
hsa04270:Vascular smooth muscle contraction	21	1.05E-09	1.33E-07
hsa05414:Dilated cardiomyopathy	16	4.99E-07	3.17E-05
hsa04020:Calcium signaling pathway	22	6.01E-07	2.54E-05
hsa05410:Hypertrophic cardiomyopathy (HCM)	14	6.23E-06	1.98E-04
hsa04610:Complement and coagulation cascades	12	2.21E-05	5.60E-04
hsa04510:Focal adhesion	18	5.90E-04	1.24E-02
hsa04512:ECM-receptor interaction	10	2.47E-03	4.39E-02
GO-BP term	Count	P-value	Benjamini
GO:0007517~muscle organ development	30	3.16E-12	7.05E-09
GO:0007155~cell adhesion	56	1.38E-11	1.54E-08
GO:0022610~biological adhesion	56	1.45E-11	1.08E-08
GO:0030198~extracellular matrix organization	20	1.34E-10	7.47E-08
GO:0006936~muscle contraction	23	5.82E-10	2.60E-07
GO:0003012~muscle system process	24	6.36E-10	2.37E-07
GO:0043062~extracellular structure organization	23	1.99E-09	6.34E-07
GO:0010033~response to organic substance	51	9.18E-09	2.56E-06
GO:0044057~regulation of system process	30	2.85E-08	7.07E-06
GO:0006875~cellular metal ion homeostasis	23	6.17E-08	1.38E-05
GO:0003018~vascular process in circulatory system	13	8.38E-08	1.70E-05
GO:0009611~response to wounding	40	9.53E-08	1.77E-05
GO:0035295~tube development	24	1.15E-07	1.98E-05
GO:0055065~metal ion homeostasis	23	1.37E-07	2.19E-05
GO:0042127~regulation of cell proliferation	51	1.50E-07	2.24E-05
GO:0009719~response to endogenous stimulus	33	3.03E-07	4.23E-05
GO:0048878~chemical homeostasis	38	3.32E-07	4.35E-05
GO:0050801~ion homeostasis	33	3.79E-07	4.70E-05
GO:0008015~blood circulation	21	4.97E-07	5.83E-05
GO:0003013~circulatory system process	21	4.97E-07	5.83E-05

KEGG, Kyoto Encyclopedia of Genes and Genomes; GO-BP, Gene Ontology-Biological Process; Count, no. of genes in term; p-value, modified Fisher exact p-value, EASE score; Benjamini, Benjamini-Hochberg corrected p-value.

PPI Networks for Differentially Expressed Genes

PPI networks of differentially expressed genes were constructed using STRING version 11.0. This PPI networks for upregulated genes consisted of 147 nodes and 1351 edges, while downregulated genes consisted of 524 nodes and 453 edges. Average node degree for upregulated genes was 18.4, while it was 1.73 for downregulated genes.

STRING database provided information on gene annotations to pathway terms (Table 3). The protein products of upregulated genes that functioned in cell cycle pathway KEGG term were selected for further analysis (Figure 1).

Figure 1. PPI network of differentially upregulated genes. Red nodes showed proteins annotated in cell cycle pathway term.

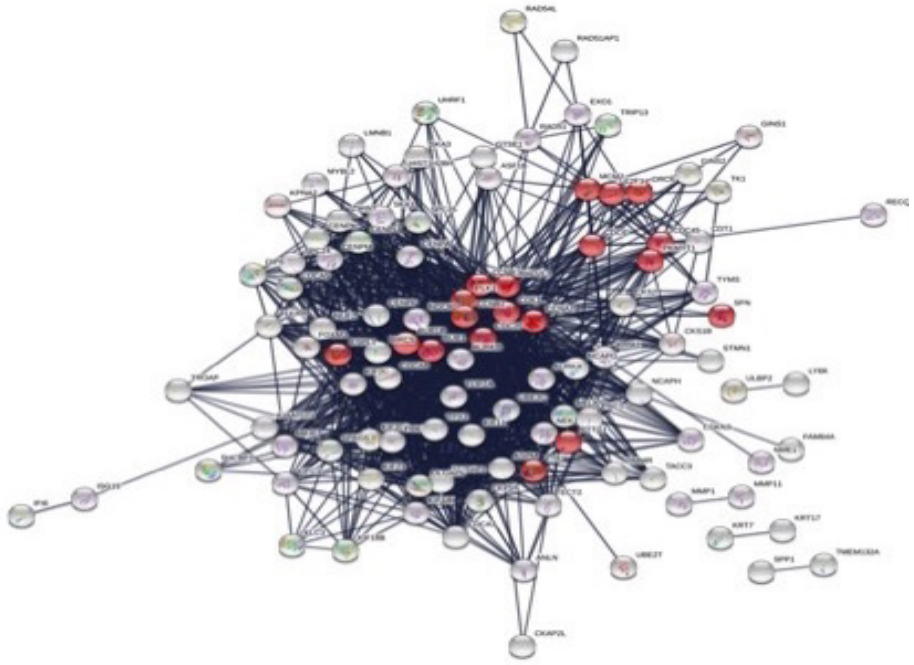

Table 3. KEGG pathway annotations of differentially expressed upregulated genes in bladder urothelial carcinoma from STRING database (FDR<0.05).

KEGG pathway term	FDR	Proteins in network
hsa04110: Cell cycle	7.81E-17	BUB1, BUB1B, CCNA2, CCNB1, CCNB2, CDC20, CDC45, CDC6, CDK1, E2F1, ESPL1, MAD2L1, MCM2, ORC6, PKMYT1, PLK1, PTTG1, SFN, TTK
hsa04114: Oocyte meiosis	1.17E-07	AURKA, BUB1, CCNB1, CCNB2, CDC20, CDK1, ESPL1, MAD2L1, PKMYT1, PLK1, PTTG1
hsa04914: Progesterone-mediated oocyte maturation	2.08E-06	AURKA, BUB1, CCNA2, CCNB1, CCNB2, CDK1, MAD2L1, PKMYT1, PLK1
hsa04115: p53 signaling pathway	3.80E-04	CCNB1, CCNB2, CDK1, GTSE1, RRM2, SFN
hsa04218: Cellular senescence	3.60E-03	CCNA2, CCNB1, CCNB2, CDK1, E2F1, FOXM1, MYBL2
hsa05166: HTLV-I infection	4.32E-02	BUB1B, CCNB2, CDC20, E2F1, MAD2L1, MYBL2, PTTG1

KEGG, Kyoto Encyclopedia of Genes and Genomes; FDR, False discovery rate

Survival Analysis of Cell Cycle Proteins

The effect of increased expression of the proteins, which functioned in cell cycle pathway, on overall patient survival was investigated to find potential prognostic biomarkers of bladder urothelial carcinoma.

18 genes were common in cell cycle KEGG pathway term in DAVID and STRING annotations, yet STRING results gave also ORC6 in KEGG pathway. The effect of total 19 proteins from DAVID and STRING annotations from cell cycle pathway on patient survival was investigated and six proteins have been found to be associated with overall survival in bladder carcinoma (Figure 2A-F).

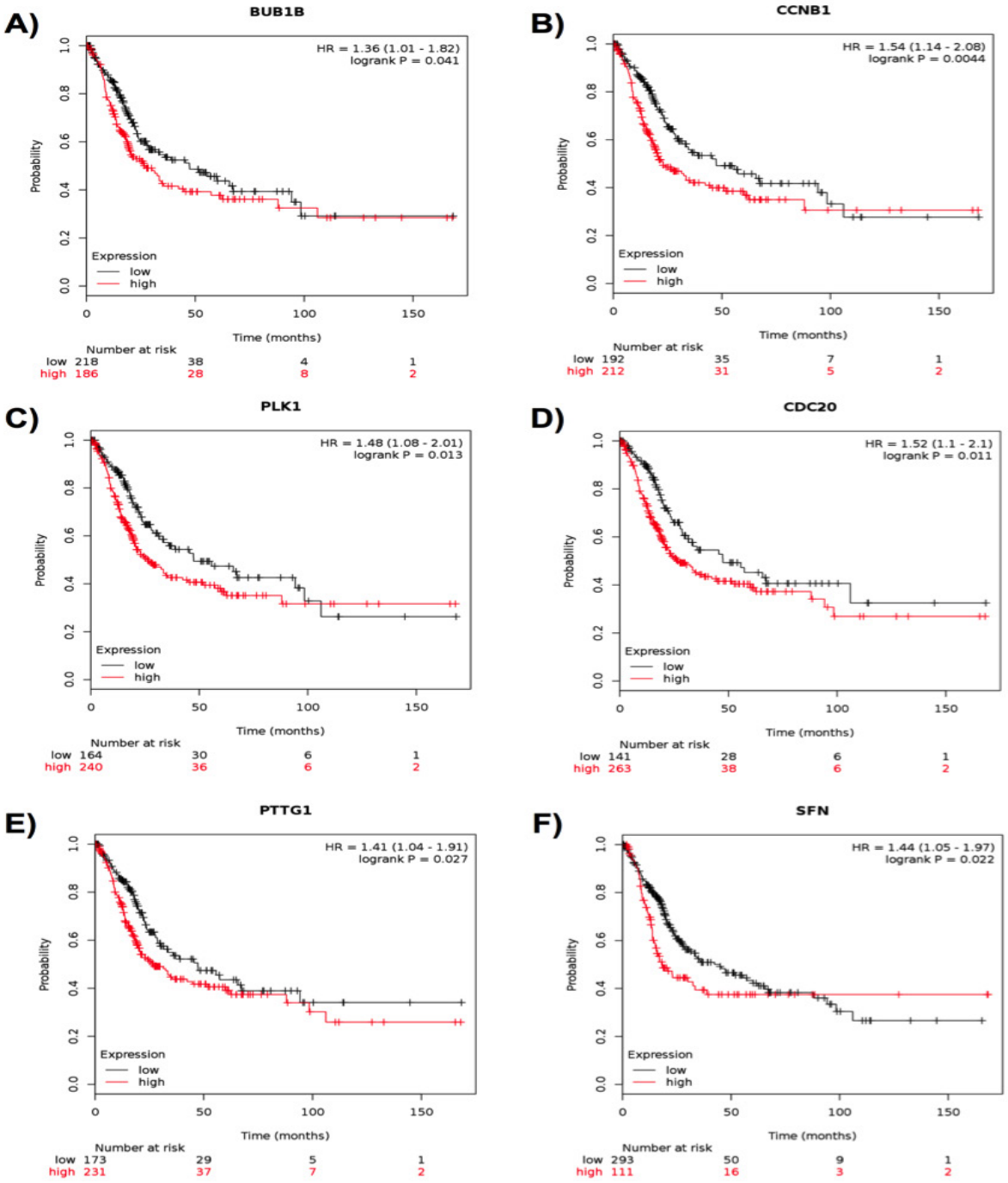

Figure 2. Kaplan-Meier survival plots showing overall survival for A) BUB1B, B) CCNB1, C) PLK1, D) CDC20, E) PTTG1 and F) SFN.

DISCUSSION

In this study, molecular mechanisms and potential prognostic biomarkers of bladder urothelial carcinoma were investigated. RNA-seq gene expression analysis was performed with high number of bladder urothelial carcinoma and normal samples. GO-BP and KEGG pathway analyses was performed, PPI networks were constructed for differentially expressed upregulated and downregulated genes. The effect of gene expression alterations of cell cycle KEGG pathway proteins on patient survival was also explored.

Here, upregulated genes mainly annotated to cell cycle pathway and cell cycle related GO-BP terms (Table 1). Annotation of upregulated genes to cell cycle pathway and cell cycle related GO-BP terms might indicate the increased mitosis and cell division in bladder urothelial carcinoma cells. The upregulation of genes in cell division terms is consistent with previous report showing enrichment of differentially expressed upregulated genes in cell division and cell cycle related terms in bladder cancer [5]. According to KEGG pathway and GO-BP term annotations, downregulated genes in bladder urothelial carcinoma cells mostly annotated to adhesion, ECM-receptor interaction, cardiomyopathy related pathways, muscle related and circulatory system related GO-BP terms (Table 2). Alteration of ECM and cell adhesion has been shown to be important processes in tumor invasion and metastasis for several cancer types [19]. Interestingly, genes related with cardiomyophaty related pathways were found to be downregulated (Table 2). The appearance of cardiomyopathy here may be related with patient history, since different chemotherapeutics have been shown to have cardiotoxicity potential [20].

In this study, the association of increased expression of 19 genes that were annotated to cell cycle pathway with patient prognosis was investigated and high expression of 6 cell cycle pathway genes were found to be associated with bladder cancer patient prognosis (Figure 2A-F). Increased CCNB1 (cyclin B1) expression and tumor growth due to CCNB1 have been shown in cancers, such as hepatocellular carcinoma and colorectal cancer [21, 22]. Overexpression of CDC20 (cell division cycle 20) has been observed in cancers and CDC20 proposed as marker for ovarian cancer [23, 24]. CDC20 and CCNB1 were previously associated with patient prognosis in bladder cancers [25], which confirms the results presented in this study (Figure 2B and Figure 2D). Increased expression of BUB1B (BUB1 mitotic checkpoint serine/threonine kinase B) has been shown in breast cancer and proposed as a potential marker for breast cancer classification [26]. Increased PLK1 (polo like kinase 1) and PTTG1 (PTTG1 regulator of sister chromatid separation, securin) expressions were detected in different bladder cancer cell lines and tissues [27-29]. The expression alteration of SFN (Stratifin or 14-3-3 protein sigma) has not been studied in bladder cancer in detail, although genetic variation and methylation status of SFN were studied in bladder cancer [30, 31]. Here, in addition to showing increased expression of PTTG1, SFN, BUB1B and PLK1 in bladder carcinoma patients (Figure 1), expression of these proteins was shown to be associated with patient survival (Figure 2).

The results presented in this study showed the altered molecular mechanisms in bladder urothelial carcinoma patients. Moreover, the prognostic and predictive value of gene products of upregulated genes that were annotated to cell cycle pathway by DAVID and STRING was investigated and the expression of six genes were associated with patient survival.

CONFLICT of INTEREST

There is no conflict of interest. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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