The expression patterns of APC2 and APC7 in newly diagnosed acute lymphoblastic leukemia

ABSTRACT

Acute lymphoblastic leukemia (ALL) is a heterogeneous type of disease that is currently categorized based on cell morphology, immunophenotype, genetic abnormalities and gene expression pattern. Although these classifications are valuable in the determination of patient's survival and treatment intensity, the response of patients to treatment and subsequently their survival are highly different, even in each subtype. So searching for new molecules involved in the leukemogenesis, disease progression, treatment resistance or candidate targets for therapy are critically sensed. APC/C is a multi-subunit E3 ligase that has essential role in metaphase progression and seems to be essentially involved in tumorgenesis and cancer progression. We analyzed the expression of APC2 and APC7 gene as two key subunits of this complex in 57 newly diagnosed ALL patients with quantitive RT-PCR. APC2 and APC7 were significantly over-expressed in 33(57.9%) and 38(66.7%) of patients respectively (P value of 0.014 and 0.009) using two-tailed Student's t tests. This over expression was independent of cellular, immunological and molecular factors. APC/C promotes cell proliferation, a feature related to tumorgenesis and also poor prognosis in cancers such as ALL, so the determination of the pattern of APC/C subunits gene expression may help to better understand molecular basic underlying cancer and also new prognostic marker and new targets for therapy in ALL patients.

Keywords: Cancer, Cell proliferation, Diagnosis, Leukemogenesis.

Introduction

Acute lymphoblastic leukemia (ALL) occurs due to successive mutations in genes that regulate vital cellular functions including self-renewal, proliferation, differentiation and apoptosis. Leukemic cell division in ALL patients require more time than normal counterparts due to a lag in the S phase progression but the rate of self-renewal and resistance to cell death is higher in these cells which gives them a chance to successfully compete with normal cells, occupy the bone marrow space and disrupt the normal hematopoiesis. Today new ALL treatment protocols consist of corticosteroid in combination with chemotherapeutic agents. These regimens mainly target microtubules assembly or DNA synthesis as blind spots of leukemic cells. These strategies enhance the cure rate of ALL patient from 10% in 1960s to 90% in children and 40% in adult patients in 2009. Nevertheless the early and late side effects of these treatment protocols in children and their low efficiency in adults are main drawbacks of these approaches. Although Abnormalities in master regulators of interphase including Rb, p16, p53, and p15 have been well documented in ALL patients, metaphase regulators are less investigated yet. Anaphase promoting Complex/Cyclosome (APC/C) is the main synchronizer of the cell cycle of G1 and metaphase (1). This protein ligase complex is composed of 19 subunits consisting three sub-complexes (TPR lobe, catalytic core, and

scaffolding platform). The complex activity begins after assembly with its coactivators including Cdh1 and Cdc20 in G1 and metaphase respectively. APC^{Cdh1} causes geminin degradation to ensure that DNA duplication occurs once and only once in each cell division (1). APC^{Cdc20} causes mitosis exit through targeting Cyclin B and Securin degradation. Occurrence of these events in a correct spatiotemporal spite of the cell cycle is necessary for the fidelity of daughter cells genome content. APC/C have many functions beyond its role in the cell cycle, it regulates stem cells self-renewal, differentiation, apoptosis, senescence and energy metabolism. It is postulated that APC/C complex dysregulation have role in tumorigenesis either in solid tumors or hematologic malignancies mainly by provoking chromosomal instabilities (1). Aberrant expression of APC/C subunits have been observed in a variety of human cancers such as breast, colon cancer and acute myeloblastic leukemia .On the other hand, it has been shown that APC/C inhibitors, such as pro-TAME, Apcin and Withaferin A, induce cell death in dividing cancerous cells. Studies on these inhibitors revealed that targeting mitotic exit regulators as a therapeutic targets lead to more efficient mitotic arrest than microtubule inhibitors such as vincristine. These agents target APC/C in a direct and consistent manner while microtubule inhibitors inhibit APC/C incompletely that can lead to mitotic slippage of some cancerous cells caused by remainder APC^{Cdc20} activity. Due to the importance of the APC complex, it is logical to investigate further the role of APC complexes. In this context, we decided to study the gene expression level of APC2 and APC7, respectively belonging to catalytic and scaffold platform sub-complex of APC/C, as two key subunits of APC/C complexes, in ALL patients in comparison with normal subjects. This evaluation may give us an insight about mitotic exit regulators status in ALL that may help us to design new strategies in monitoring and treatment of patients.

Material and methods

Patients

A total 57 peripheral blood (PB) and bone marrow (BM) samples at the time of diagnosis and before any chemotherapy was given, were obtained from ALL patients between July 2014 and September 2016. Specimens were collected from all patients with informed consent in agreement with the Declaration of Helsinki (1). Diagnosis was made according to PB or BM film, immunophenotyping and molecular examination. Immunophenotypic analysis was based on EGIL classification (2). Due to the limited number of T-lineage ALL patients, sub-classification of this group do not inter in statistical analysis. Demographic and subclinical characteristics of patients samples are summarized in Table 2. Eleven PB or BM samples were obtained from normal subjects as control group.

RNA Extraction and cDNA synthesis

Mononuclear cells were isolated from PB or BM samples with Ficoll-Hypaque density gradient centrifugation and immediately mixed with 1ml of trizol reagent in order to inhibit nucleic acid degradation by RNase and DNase. These specimens were immediately cryopreserved or prepared for RNA extraction. Total RNA was extracted from 1ml of each

specimens, according to the single-step method (1). Quantity and quality of total RNA and contamination with genomic DNA were exanimated by Nanodrop and agarose gel electrophoresis. RNA to cDNA conversion was performed according to ABI manuscript by AMV RT enzyme.

Analysis of gene expression by quantitative real-time PCR

Real-time PCR primers for target genes and housekeeping gene were designed using gene runner x64 v 6.0.28 beta (primers properties are summarized in table 1) and primer specifity was verified by NCBI primer-blast tool. A SYBR Green I Real-time PCR assay was performed in 25µl final reaction volume using 5µl cDNA (100ng RNA equivalent), 0.75µl primers (300nM), 12.5 universal Master Mix, 2.5µl PCR buffer 10X and sterilized distilled water to reach total volume. Thermal cycling was carried out on ABI thermocycler, using the following cycling conditions: 10 min at $95^{O}C$, then, followed by 40 cycles at $95^{O}C$ for 15 s and $60^{O}C$ for 30 s. Efficiency of all primer were setup by triplicate testing of five serial dilutions of cDNA at 0.95-0.99.ΔCT was calculated from $C_{T,\ target\ genes}$-$C_{T,\ ABL}$ formula and $2^{-\Delta Ct,\ case}/2^{-\Delta Ct,\ control}$ was considered as gene expression fold changes[44].

Table 1. Real-Time PCR oligonucleotide primers

Gene		Sequence	TM(^{o}C)	Amplicon(number of nucleotides)
APC2	APC2F	CAGCTCAGCCAGGTCTTACACAG	60.1	199
	APC2	CGTCCTGCAGGAACACCTTG	60.3	
APC7	APC7F	ACCCTGAGTTATTCTCCC	52.3	100
	APC7	TACTTACTCACAGCATTCCG	54.9	
ABL	ABLF	TGGAGATAACACTCTAAGCATAACTA	59.1	124
	ABLR	GATGTAGTTGCTTGGGACCCA	60.0	

Statistical analysis

Data are expressed by mean± SD. All tests were done in duplicate and the mean of CV was 0.71% that show a good inter-run reproducibility for RT-PCR assay. According to Levene´s test and Shapiro-Wilk test results we used from One-Way ANOVA or Kruskal-Wallis for multi-state variables and t-test or Mann-Whitney U test for two-state variables. For analysis correlation, Pearson´s test was performed. Two tailed P value less than 0.05 was considered significant.

Results

Overall we studied 57 patients with acute lymphoblastic leukemia at the time of diagnosis in the range of 1-81 years old (median, 21years). The prevalence of recurrent of ALL associated translocations were 6(10.53%), 3(5.26%), 3(5.26%) and 1(1.75%) for t(12;21), t(9;22), t(1;19), and t(4;11) respectively (table 2). APC2 and APC7 gene expression levels were not significantly correlated with the types of samples (BM or PB), immunological categories (EGIL classification), gender, age and translocation variable (Figure 1). APC2 and APC7 were significantly over-expressed in patients samples with a two-tailed Student's t tests P value of 0.014 and 0.009 for these genes respectively. The normalized expression ratio was 6.93 and 6.88 for APC2 and APC7 respectively (Figure 2). APC2 and APC7 overexpression were seen in 33(57.9%) and 38(66.7%) patients. In 24(42.15%) patients the level of APC2 and APC7 were significantly over-expressed simultaneously.

Table 2. Summary of patient's demographic data

Study population(N=57)	
Age, y (median, y)	21(1-81)
Sex(Male/Female)	29/28
Sample type(Peripheral blood/Bone marrow)	10/47
Blast percent(Peripheral blood/Bone marrow)	74.2/75.3
Translocation(Positive/Negative)	13/44
t(12;21)	6
t(9;22)	3
t(1;19)	3
t(4;11)	1
Immunological Classification (%)	
Pro-B ALL	8(14%)
Common-B ALL	22(38.6%)
Pre-B ALL	14(24.6%)
Mature-B ALL	7(12.3%)
T- lineage ALL	6(10.5%)
APC2 Expression(over-Expression/Normal)	33(57.9%)/24(42.1%)
APC2 Expression(over Expression/Normal)	38(66.7%)/19(33.3%)

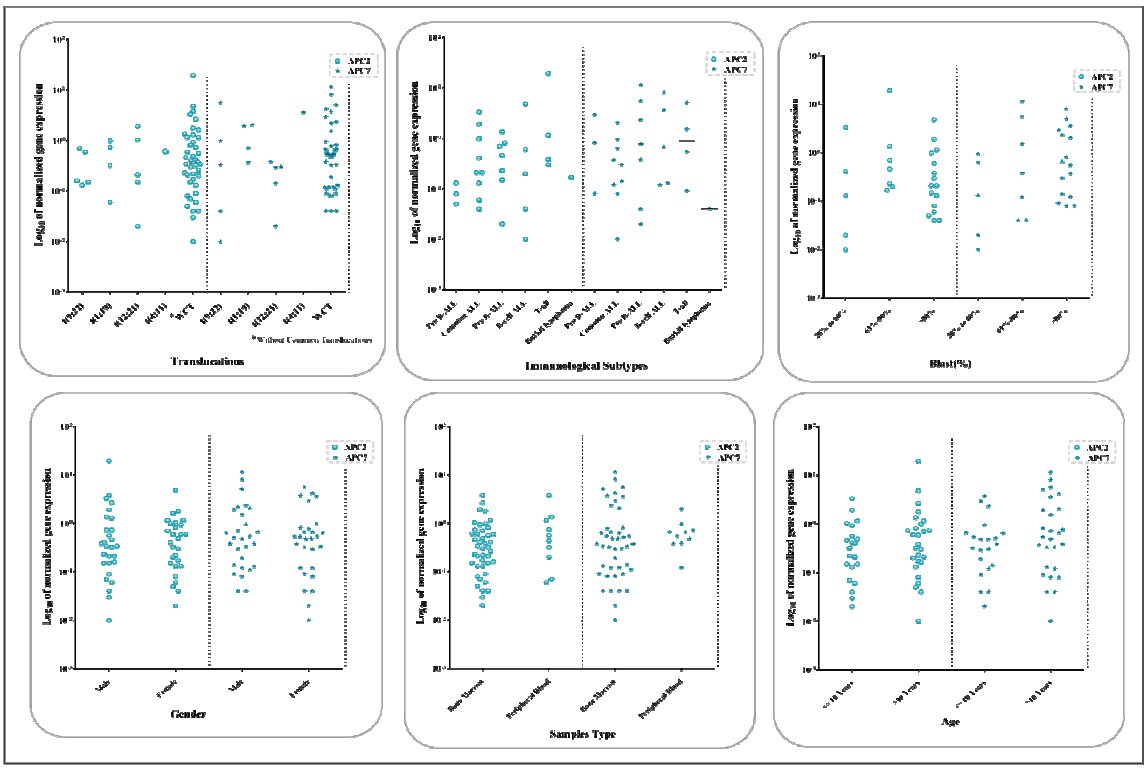

Figure 1. APC/C subunits gene expression level in ALL patients

Gene expression levels of APC2 and APC7 were compared between different sub-groups of ALL samples. P values of multi variables sub-groups (including the type of translocations, immunological sub-types and blast percent) were calculated by Kruskal–Wallis test and for two variables(gender, sample type and age) by Mann-Whitney U test(all P values were above 0.05 and insignificant(data are not shown))

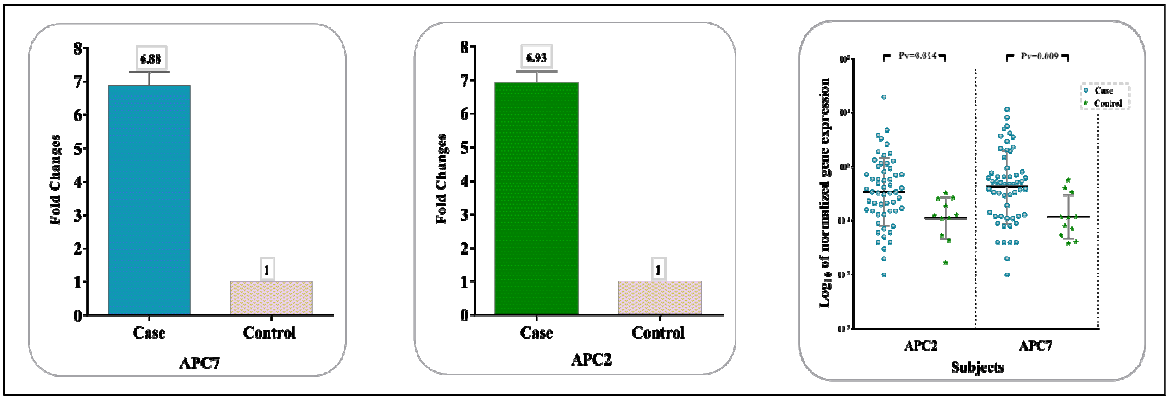

Figure 2. Quantitative RT-PCR analysis of APC2 and APC7 normalized Gene expression

Relative gene expression of APC2 and APC7 are presented by column and dot plots. APC2 and APC7 were over-expressed 6.88 and 6.93 fold in ALL patients than control samples

respectively. These overexpression was seen in 33(57.9%) and 38(66.7%) of patients respectively (P value of 0.014 and 0.009) using two-tailed Student's t tests.

Discussion

ALL is a heterogeneous type of disease with different molecular, biological, immunophenotypic and morphological subtypes. In ALL patients, response to available therapeutic regimens is markedly variable even in each subtype. Resistant patients may need new therapeutic strategies designed based on underlying mechanisms of the cancers. This aim is feasible with the investigation about molecular mechanisms behind the formation of cancer cells. In this regard, cell cycle regulators are at the focus center. APC/C, as a critical cell cycle regulator, seems to be important in cancer formation and progression (3). Mutations in the subunits of the APC/C complex have been documented in many types of cancers including breast cancer, colon cancer, glioma, and hepatocarcinoma. Recent studies also have shown increased APC/C subunits/ activator expression in a variety of solid tumors and hematologic malignancies. Other studies demonstrated a significant correlation between APC/C levels of activation and disease prognosis. Our results showed a statistically significant increased in the levels of APC2 and APC7 expression in ALL patients, but it was not significantly correlated with immunological subtypes of ALL, chromosomal translocation, FAB classification, gender, blast percent and the age of the patients.

In agreement with our findings, over expression of APC2 and APC7 has been previously reported in AML patients [15].This over expression has been also documented in cell lines with hematologic (RPMI and CCRF-CEM) or solid tumor origins. However, some studies have shown both APC7 down-regulation and over-expression in different forms of breast cancer [29,30] which reflects a context dependent manner of APC/C function in this cancer. As we know APC/C over activation, either by increased subunits expression or post translational activation is correlated with high-rate of cell proliferation, increased proliferation rapidity is significantly associated with poor prognosis in ALL patients. Thus it is possible that APC/C subunits expression is also an independent prognostic marker in leukemic patients but it need to be proofed using further clinical studies.

In the field of chemotherapy, drugs such as vincristine induce cell death through inhibition of the microtubule assembly. Various cancer cells have different and incomplete response to vincristine based on their rate of APC complex synthesis that make it difficult to adjust treatment dose due to its severe side effects such as neuropathy [35]. APC/C inhibitors can be appropriate substitute for microtubule inhibitors as routine drugs in ALL therapy, because these agents promote mitotic arrest more efficiently than microtubule inhibitors and principally have not serious side effects on nervous system because they have not effects on microtubule assembly.

Taken together our results opened a new window to the role of mitotic exit regulatory elements in ALL tumorgenesis and transformation. Since that we proved they have aberrant pattern of expression, they may propel leukemic cells toward more proliferation.

Conclusion

The main challenge of dividing cells is duplication of 6 billion bases of DNA and accurate segregation of this DNA content between daughter cells. The fidelity of genome content during cell division is controlled in three major checkpoints. Disruption of these checkpoints is common hallmarks of human cancers. Spindle assembly checkpoint (SAC) is the main regulator of chromosome segregation in metaphase that regulates APC/C activity as an effector molecule (36-43). Overexpression of APC/C may cause decreased inhibition by SAC and subsequently may lead to chromosome missegregation and aneuploidy. Our study demonstrated that APC2 and APC7 are overexpressed simultaneously in newly cases of ALL. Accordingly, with respect to the role of APC/C in chromosomal integrity, it is not unexpected to see high rate of chromosome aberrancies such as aneuploidy and translocation in ALL leukemic blasts. So this over-activation may be involved in the initiation of malignancy and its evolution. Also APC/C over expression may promotes cell proliferation, a feature related to poor prognosis in ALL patients, so the determination of the rate of APC/C subunits expression may help us to find poor prognosis ALL patients and to better risk-stratify patients beside using the conventional risk factors.

Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Pui, C.H. and W.E. Evans, Acute lymphoblastic leukemia. N Engl J Med, 1998. 339(9): p. 605-15.
2. Pui, C.H., L.L. Robison, and A.T. Look, Acute lymphoblastic leukaemia. Lancet, 2008. 371(9617): p. 1030-43.
3. Campana, D. and G. Janossy, Proliferation of normal and malignant human immature lymphoid cells. Blood, 1988. 71(5): p. 1201-10.
4. Pui, C.H. and W.E. Evans, Treatment of acute lymphoblastic leukemia. N Engl J Med, 2006. 354(2): p. 166-78.
5. Bassan, R. and D. Hoelzer, Modern therapy of acute lymphoblastic leukemia. J Clin Oncol, 2011. 29(5): p. 532-43.
6. Hunger, S.P. and C.G. Mullighan, Acute Lymphoblastic Leukemia in Children. N Engl J Med, 2015. 373(16): p. 1541-52.
7. Inaba, H., M. Greaves, and C.G. Mullighan, Acute lymphoblastic leukaemia. Lancet, 2013. 381(9881): p. 1943-55.
8. Stock, W., et al., Cell cycle regulatory gene abnormalities are important determinants of leukemogenesis and disease biology in adult acute lymphoblastic leukemia. Blood, 2000. 95(7): p. 2364-71.
9. Sivakumar, S. and G.J. Gorbsky, Spatiotemporal regulation of the anaphase-promoting complex in mitosis. Nat Rev Mol Cell Biol, 2015. 16(2): p. 82-94.
10. Chang, L., et al., Atomic structure of the APC/C and its mechanism of protein ubiquitination. Nature, 2015. 522(7557): p. 450-4.
11. Sivaprasad, U., Y.J. Machida, and A. Dutta, APC/C--the master controller of origin licensing? Cell Div, 2007. 2: p. 8.

12. Derive, N., et al., Bub3-BubR1-dependent sequestration of Cdc20Fizzy at DNA breaks facilitates the correct segregation of broken chromosomes. J Cell Biol, 2015. 211(3): p. 517-32.
13. Zhou, Z., et al., Insights into APC/C: from cellular function to diseases and therapeutics. Cell Div, 2016. 11: p. 9.
14. Wasch, R. and D. Engelbert, Anaphase-promoting complex-dependent proteolysis of cell cycle regulators and genomic instability of cancer cells. Oncogene, 2005. 24(1): p. 1-10.
15. Rahimi, H., et al., The expression pattern of APC2 and APC7 in various cancer cell lines and AML patients. Adv Med Sci, 2015. 60(2): p. 259-63.
16. Wang, L., et al., Targeting Cdc20 as a novel cancer therapeutic strategy. Pharmacol Ther, 2015. 151: p. 141-51.
17. Zeng, X., et al., Pharmacologic inhibition of the anaphase-promoting complex induces a spindle checkpoint-dependent mitotic arrest in the absence of spindle damage. Cancer Cell, 2010. 18(4): p. 382-95.
18. Huang, H.C., et al., Evidence that mitotic exit is a better cancer therapeutic target than spindle assembly. Cancer Cell, 2009. 16(4): p. 347-58.
19. Association, W.M., World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects. Jama, 2013. 310(20): p. 2191-4.
20. Bene, M.C., et al., Proposals for the immunological classification of acute leukemias. European Group for the Immunological Characterization of Leukemias (EGIL). Leukemia, 1995. 9(10): p. 1783-6.
21. Chomczynski, P. and N. Sacchi, The single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction: twenty-something years on. Nat Protoc, 2006. 1(2): p. 581-5.
22. Schmittgen, T.D. and K.J. Livak, Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc, 2008. 3(6): p. 1101-8.
23. Pui, C.H., et al., Biology, risk stratification, and therapy of pediatric acute leukemias: an update. J Clin Oncol, 2011. 29(5): p. 551-65.
24. Yeoh, E.J., et al., Classification, subtype discovery, and prediction of outcome in pediatric acute lymphoblastic leukemia by gene expression profiling. Cancer Cell, 2002. 1(2): p. 133-43.
25. Bennett, J.M., et al., Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group. Br J Haematol, 1976. 33(4): p. 451-8.
26. Ross, M.E., et al., Classification of pediatric acute lymphoblastic leukemia by gene expression profiling. Blood, 2003. 102(8): p. 2951-9.
27. Wang, Q., et al., Alterations of anaphase-promoting complex genes in human colon cancer cells. Oncogene, 2003. 22(10): p. 1486-90.
28. Lehman, N.L., et al., Oncogenic regulators and substrates of the anaphase promoting complex/cyclosome are frequently overexpressed in malignant tumors. Am J Pathol, 2007. 170(5): p. 1793-805.
29. Sivakumar S, Gorbsky GJ. Spatiotemporal regulation of the anaphase-promoting complex in mitosis. Nat Rev Mol Cell Biol. 2015;16(2):82-94.
30. Sivaprasad U, Machida YJ, Dutta A. APC/C--the master controller of origin licensing? Cell Div. 2007;2:8.
31. Kraft, C., et al., Mitotic regulation of the human anaphase-promoting complex by phosphorylation. Embo j, 2003. 22(24): p. 6598-609.
32. Kramer, E.R., et al., Mitotic regulation of the APC activator proteins CDC20 and CDH1. Mol Biol Cell, 2000. 11(5): p. 1555-69.

33. Pich, A., et al., Prognostic value of the rapidity of bone marrow blast cell proliferation in adult acute lymphoblastic leukemia. Leukemia, 2004. 18(1): p. 172-4.
34. Schneider, P., et al., The growth of highly proliferative acute lymphoblastic leukemia may be independent of stroma and/or angiogenesis. Leukemia, 2001. 15(7): p. 1143-5.
35. Ness, K.K., et al., Adverse effects of treatment in childhood acute lymphoblastic leukemia: general overview and implications for long-term cardiac health. Expert Rev Hematol, 2011. 4(2): p. 185-97.
36. Nasri H, Dehghan Shahreza F. Defensins usage as novel therapeutic and diagnostic approach. Immunopathol Persa. 2015;1(1):e05.
37. Shakweer MM, El-Sheshtawy NM. Emerging role of Treg FOXP3 expression in cancer prognosis and autoimmune diseases. Immunopathol Persa. 2017;3(1):e01.
38. Dehghan Shahreza F. From oxidative stress to endothelial cell dysfunction. J Prev Epidemiol. 2016; 1(1):e04.
39. Naqvi R. Use of rituximab in immunological disorders. Immunopathol Persa. 2016;2(1):e06
40. Salami A, Amiri M. On the occasion of world cancer day 2017; breast cancer. J Ischemia Tissue Repair. 2017;1(1):e02.
41. Nasri H. Sudden onset of renal failure requiring dialysis associated with large B-cell lymphoma of colon. J Nephropathol. 2012 Oct;1(3):202-6.
42. Balwani MR, Kute VB, Shah PR, Wakhare P, Trivedi HL. Secondary renal amyloidosis in a patient of pulmonary tuberculosis and common variable immunodeficiency. J Nephropharmacol. 2015 Jun 21;4(2):69-71.
43. Yaghoubi F, Yarmohammadi M, Vasei M. Paraneoplastic proteinuria in papillary renal cell carcinoma; a case report. J Renal Inj Prev. 2016 Aug 3;5(4):207-9.
44. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nature protocols. 2008;3(6):1101-8.