# 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

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

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## Material and methods

### Patients

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

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### RNA Extraction and cDNA synthesis

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

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

87 **Analysis of gene expression by quantitative real-time PCR**

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

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**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 | |

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101 **Statistical analysis**

102    Data are expressed by mean± SD. All tests were done in duplicate and the mean of CV was
103    0.71% that show a good inter-run reproducibility for RT-PCR assay. According to Levene´s
104    test and Shapiro-Wilk test results we used from One-Way ANOVA or Kruskal-Wallis for
105    multi-state variables and t-test or Mann-Whitney U test for two-state variables. For analysis
106    correlation, Pearson´s test was performed. Two tailed P value less than 0.05 was considered
107    significant.

108

109 **Results**

110 Overall we studied 57 patients with acute lymphoblastic leukemia at the time of diagnosis in

111 the range of 1-81 years old (median, 21years). The prevalence of recurrent of ALL associated

112 translocations were 6(10.53%), 3(5.26%), 3(5.26%) and 1(1.75%) for t(12;21), t(9;22),

113 t(1;19), and t(4;11) respectively (table 2). APC2 and APC7 gene expression levels were not

114 significantly correlated with the types of samples (BM or PB), immunological categories

115 (EGIL classification), gender, age and translocation variable (Figure 1). APC2 and APC7

116 were significantly over-expressed in patients samples with a two-tailed Student's t tests P

117 value of 0.014 and 0.009 for these genes respectively. The normalized expression ratio was

118 6.93 and 6.88 for APC2 and APC7 respectively (Figure 2). APC2 and APC7 overexpression

119 were seen in 33(57.9%) and 38(66.7%) patients. In 24(42.15%) patients the level of APC2

120 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%) |

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

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

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

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

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

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

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

172

173 **Conclusion**

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

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190 **Conflict of interest**

191 The authors declare that they have no conflict of interest.

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194 **References**
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196 1.    Pui, C.H. and W.E. Evans, Acute lymphoblastic leukemia. N Engl J Med, 1998.
197       339(9): p. 605-15.
198 2.    Pui, C.H., L.L. Robison, and A.T. Look, Acute lymphoblastic leukaemia. Lancet,
199       2008. 371(9617): p. 1030-43.
200 3.    Campana, D. and G. Janossy, Proliferation of normal and malignant human immature
201       lymphoid cells. Blood, 1988. 71(5): p. 1201-10.
202 4.    Pui, C.H. and W.E. Evans, Treatment of acute lymphoblastic leukemia. N Engl J
203       Med, 2006. 354(2): p. 166-78.
204 5.    Bassan, R. and D. Hoelzer, Modern therapy of acute lymphoblastic leukemia. J Clin
205       Oncol, 2011. 29(5): p. 532-43.
206 6.    Hunger, S.P. and C.G. Mullighan, Acute Lymphoblastic Leukemia in Children. N
207       Engl J Med, 2015. 373(16): p. 1541-52.
208 7.    Inaba, H., M. Greaves, and C.G. Mullighan, Acute lymphoblastic leukaemia. Lancet,
209       2013. 381(9881): p. 1943-55.
210 8.    Stock, W., et al., Cell cycle regulatory gene abnormalities are important determinants
211       of leukemogenesis and disease biology in adult acute lymphoblastic leukemia. Blood,
212       2000. 95(7): p. 2364-71.
213 9.    Sivakumar, S. and G.J. Gorbsky, Spatiotemporal regulation of the anaphase-
214       promoting complex in mitosis. Nat Rev Mol Cell Biol, 2015. 16(2): p. 82-94.
215 10.   Chang, L., et al., Atomic structure of the APC/C and its mechanism of protein
216       ubiquitination. Nature, 2015. 522(7557): p. 450-4.
217 11.   Sivaprasad, U., Y.J. Machida, and A. Dutta, APC/C--the master controller of origin
218       licensing? Cell Div, 2007. 2: p. 8.

219   12.   Derive, N., et al., Bub3-BubR1-dependent sequestration of Cdc20Fizzy at DNA
220         breaks facilitates the correct segregation of broken chromosomes. J Cell Biol, 2015.
221         211(3): p. 517-32.
222   13.   Zhou, Z., et al., Insights into APC/C: from cellular function to diseases and
223         therapeutics. Cell Div, 2016. 11: p. 9.
224   14.   Wasch, R. and D. Engelbert, Anaphase-promoting complex-dependent proteolysis of
225         cell cycle regulators and genomic instability of cancer cells. Oncogene, 2005. 24(1):
226         p. 1-10.
227   15.   Rahimi, H., et al., The expression pattern of APC2 and APC7 in various cancer cell
228         lines and AML patients. Adv Med Sci, 2015. 60(2): p. 259-63.
229   16.   Wang, L., et al., Targeting Cdc20 as a novel cancer therapeutic strategy. Pharmacol
230         Ther, 2015. 151: p. 141-51.
231   17.   Zeng, X., et al., Pharmacologic inhibition of the anaphase-promoting complex induces
232         a spindle checkpoint-dependent mitotic arrest in the absence of spindle damage.
233         Cancer Cell, 2010. 18(4): p. 382-95.
234   18.   Huang, H.C., et al., Evidence that mitotic exit is a better cancer therapeutic target than
235         spindle assembly. Cancer Cell, 2009. 16(4): p. 347-58.
236   19.   Association, W.M., World Medical Association Declaration of Helsinki: ethical
237         principles for medical research involving human subjects. Jama, 2013. 310(20): p.
238         2191-4.
239   20.   Bene, M.C., et al., Proposals for the immunological classification of acute leukemias.
240         European Group for the Immunological Characterization of Leukemias (EGIL).
241         Leukemia, 1995. 9(10): p. 1783-6.
242   21.   Chomczynski, P. and N. Sacchi, The single-step method of RNA isolation by acid
243         guanidinium thiocyanate-phenol-chloroform extraction: twenty-something years on.
244         Nat Protoc, 2006. 1(2): p. 581-5.
245   22.   Schmittgen, T.D. and K.J. Livak, Analyzing real-time PCR data by the comparative
246         C(T) method. Nat Protoc, 2008. 3(6): p. 1101-8.
247   23.   Pui, C.H., et al., Biology, risk stratification, and therapy of pediatric acute leukemias:
248         an update. J Clin Oncol, 2011. 29(5): p. 551-65.
249   24.   Yeoh, E.J., et al., Classification, subtype discovery, and prediction of outcome in
250         pediatric acute lymphoblastic leukemia by gene expression profiling. Cancer Cell,
251         2002. 1(2): p. 133-43.
252   25.   Bennett, J.M., et al., Proposals for the classification of the acute leukaemias. French-
253         American-British (FAB) co-operative group. Br J Haematol, 1976. 33(4): p. 451-8.
254   26.   Ross, M.E., et al., Classification of pediatric acute lymphoblastic leukemia by gene
255         expression profiling. Blood, 2003. 102(8): p. 2951-9.
256   27.   Wang, Q., et al., Alterations of anaphase-promoting complex genes in human colon
257         cancer cells. Oncogene, 2003. 22(10): p. 1486-90.
258   28.   Lehman, N.L., et al., Oncogenic regulators and substrates of the anaphase promoting
259         complex/cyclosome are frequently overexpressed in malignant tumors. Am J Pathol,
260         2007. 170(5): p. 1793-805.
261   29.   Sivakumar S, Gorbsky GJ. Spatiotemporal regulation of the anaphase-promoting
262         complex in mitosis. Nat Rev Mol Cell Biol. 2015;16(2):82-94.
263   30.   Sivaprasad U, Machida YJ, Dutta A. APC/C--the master controller of origin
264         licensing? Cell Div. 2007;2:8.
265   31.   Kraft, C., et al., Mitotic regulation of the human anaphase-promoting complex by
266         phosphorylation. Embo j, 2003. 22(24): p. 6598-609.
267   32.   Kramer, E.R., et al., Mitotic regulation of the APC activator proteins CDC20 and
268         CDH1. Mol Biol Cell, 2000. 11(5): p. 1555-69.

269    33.    Pich, A., et al., Prognostic value of the rapidity of bone marrow blast cell proliferation
270          in adult acute lymphoblastic leukemia. Leukemia, 2004. 18(1): p. 172-4.
271    34.    Schneider, P., et al., The growth of highly proliferative acute lymphoblastic leukemia
272          may be independent of stroma and/or angiogenesis. Leukemia, 2001. 15(7): p. 1143-
273          5.
274    35.    Ness, K.K., et al., Adverse effects of treatment in childhood acute lymphoblastic
275          leukemia: general overview and implications for long-term cardiac health. Expert Rev
276          Hematol, 2011. 4(2): p. 185-97.
277    36. Nasri H, Dehghan Shahreza F. Defensins usage as novel therapeutic and diagnostic
278          approach. Immunopathol Persa. 2015;1(1):e05.
279    37. Shakweer MM, El-Sheshtawy NM. Emerging role of Treg FOXP3 expression in cancer
280          prognosis and autoimmune diseases. Immunopathol Persa. 2017;3(1):e01.
281    38. Dehghan Shahreza F. From oxidative stress to endothelial cell dysfunction. J Prev
282          Epidemiol. 2016; 1(1):e04.
283    39.   Naqvi R. Use of rituximab in immunological disorders. Immunopathol Persa.
284          2016;2(1):e06
285    40. Salami A, Amiri M. On the occasion of world cancer day 2017; breast cancer. J Ischemia
286          Tissue Repair. 2017;1(1):e02.
287    41. Nasri H. Sudden onset of renal failure requiring dialysis associated with large B-cell
288          lymphoma of colon. J Nephropathol. 2012 Oct;1(3):202-6.
289    42. Balwani MR, Kute VB, Shah PR, Wakhare P, Trivedi HL. Secondary renal amyloidosis
290          in a patient of pulmonary tuberculosis and common variable immunodeficiency. J
291          Nephropharmacol. 2015 Jun 21;4(2):69-71.
292    43. Yaghoubi F, Yarmohammadi M, Vasei M. Paraneoplastic proteinuria in papillary renal
293          cell carcinoma; a case report. J Renal Inj Prev. 2016 Aug 3;5(4):207-9.
294    44. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T)
295          method. Nature protocols. 2008;3(6):1101-8.