

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 tumorigenesis 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 quantitative 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 tumorigenesis 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 **s** 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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66 **Material and methods**

67 **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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78 **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 examined 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 house keeping gene were designed using gene
 89 runner x64 v 6.0.28 beta (primers properties are summarized in table 1) and primer specificity
 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 sdH2O to reach
 93 total volume. Thermal cycling was carried out on ABI thermocycler, using the following
 94 cycling conditions: 10 min at 95°C, then, followed by 40 cycles at 95°C for 15 s and 60°C
 95 for 30 s. Efficiency of all primer were setup by triplicate testing of five serial dilutions of
 96 cDNA at 0.95-0.99. ΔCT was calculated from $C_{T, target genes} - C_{T, ABL}$ formula and $2^{-\Delta Ct, case} / 2^{-\Delta Ct, control}$
 97 was considered as gene expression fold changes[44].

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Table 1. Real-Time PCR oligonucleotide primers

Gene	Sequence	TM	Amplicon
APC2	APC2F CAGCTCAGCCAGGTCTTACACAG	60.1	199
	APC2 CGTCCTGCAGGAACACCTTG	60.3	
APC7	APC7F ACCCTGAGTTATTCTCCC	52.3	100
	APC7 TACTTACTCACAGCATTCCG	54.9	
ABL	ABLF TGGAGATAAACTCTAAGCATAACTA	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 test was performed. Two tailed Pvalue less than 0.05 was considered
 107 significant.

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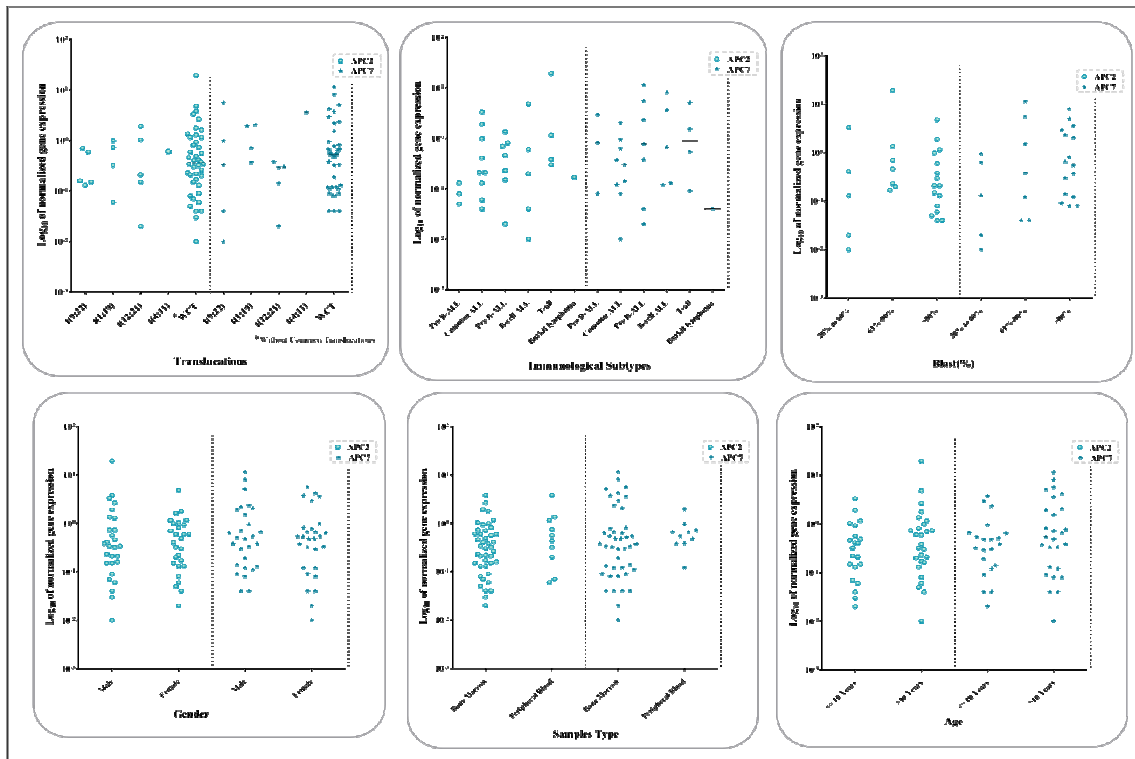
109 **Results**

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

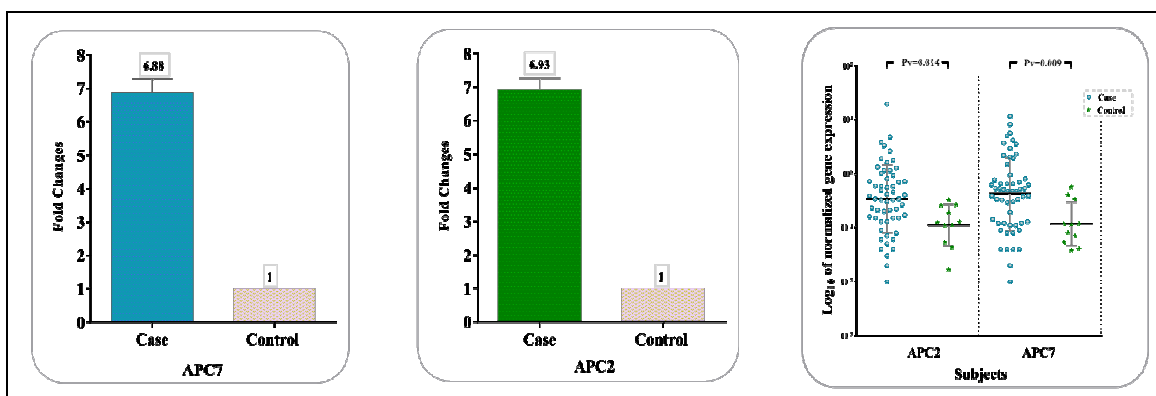
Study population(N=57)	
Age, y (median, y)	21(1-81)
Sex(Male/Female)	29/28
Sample type(Peripheral blood/Bone marrow)	10/47
Blast percent(Peripheral blood/Bone marrow)	74.2/75.3
Translocation(Positive/Negative)	13/44
t(12;21)	6
t(9;22)	3
t(1;19)	3
t(4;11)	1
Immunological Classification (%)	
Pro-B ALL	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%)
APC7 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

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))



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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

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 tumorigenesis 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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