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

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4

5 ## ABSTRACT

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

23 **Keywords:** Cancer, Cell proliferation, Diagnosis, Leukemogenesis.

24

25 ## Introduction

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

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42 scaffolding platform). The complex activity is begins after assembly with it's 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 has role in
50 tumorigenesis either solid tumors or hematologic malignancies mainly by provoking
51 chromosomal instabilities (1). Aberrant expression of APC/C subunits has been observed in a
52 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 cell caused by remainder APC^{Cdc20} activity.
59 Due to importance of APC complex, it is logical to investigate more 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.

65

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 patient between July 2014 and
70 September 2016. Specimens were collected from all patient 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 basis
73 on EGIL classification (2). Due to the limited number of T-lineage ALL patient, sub-
74 classification of this group do not inter in statistical analysis. Demographic and subclinical
75 characteristics of patients sample are summarized in Table 2. Eleven PB or BM samples were
76 obtained from normal subjects as control group.

77

78 **RNA Extraction and cDNA conversion**

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

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83 of each specimens, according to the single-step method (1). Quantity and quality of total
84 RNA and 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 house keeping 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 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 diluted cDNA
96 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
97 considered as gene expression fold changes.

98

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	TGGAGATAACACTCTAAGCATAACTA	59.1	124
	ABLR	GATGTAGTTGCTTGGGACCCA	60.0	

99

100

101 **Statistical analysis**

102 Data are expressed by mean± SD. All tests were done duplicate and the mean of CV was
103 0.71% that shown 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 analyzing
106 of correlation, Pearson test was performed. Two tailed Pvalue less than 0.05 was considered
107 as significant.

108

109 **Results**

110 In overall, we studied 57 patients with acute lymphoblastic leukemia at the time of diagnosis
111 in the range of 1-81 years (median, 21years). The prevalence of recurrent ALL associated

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112 translocation 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). The mean and SD of
116 normalized gene expression were 1.04 ± 0.35 and 1.24 ± 0.41 in patient samples and 0.15 ±
117 0.10 and 0.18 ± 0.17 in control samples for APC2 and APC7 respectively. APC2 and APC7
118 were significantly over-expressed in patients sample with a two-tailed Student's t tests P
119 value of 0.014 and 0.009 for these genes respectively. The normalized expression ratio was
120 6.93 and 6.88 for APC2 and APC7 respectively (Figure 2). APC2 and APC7 overexpression
121 were seen in 33(57.9%) and 38(66.7%) patients. In 24(42.15%) patients the level of APC2
122 and APC7 were significantly over-expressed simultaneously.

Table 2. Summary of patient's demographic data

Study population(N=57)	
Age, y (median, y)	14(1-81)
Sex(Male/Female)	28/28
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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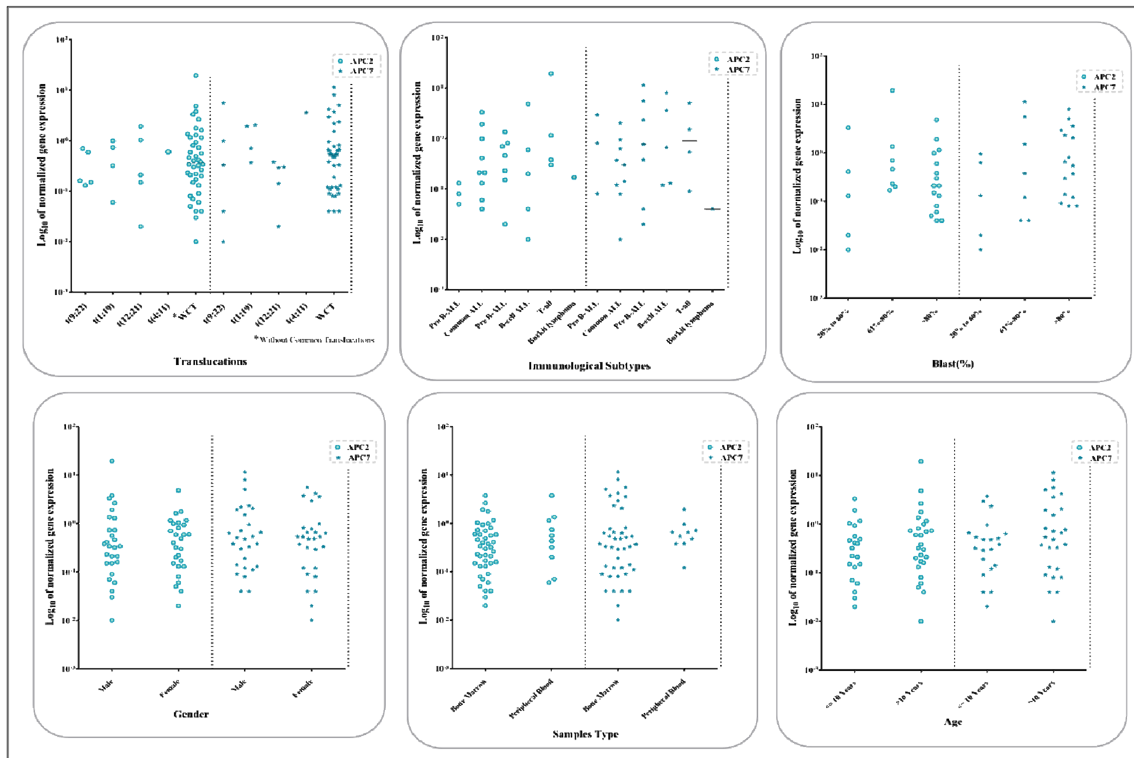

124

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

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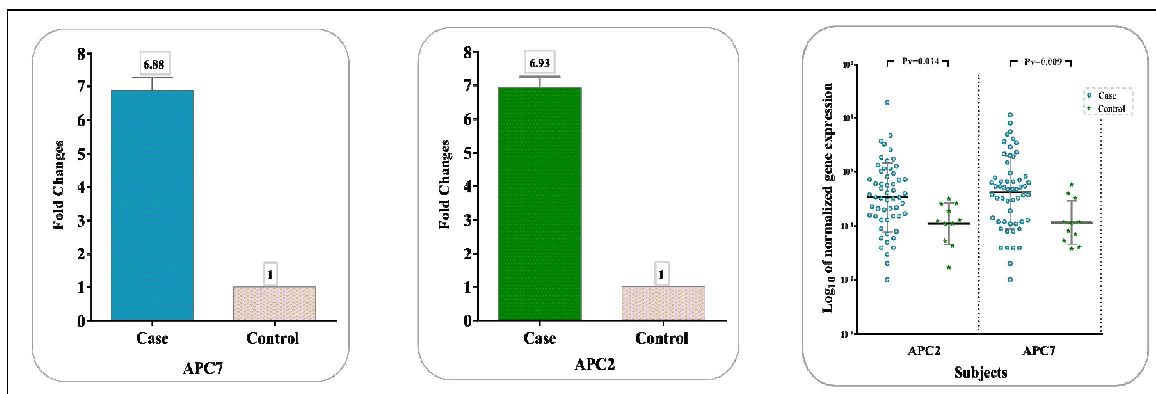

127

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

129

130 **Discussion**

131 ALL is a heterogeneous type of disease with different molecular, biological,
132 immunophenotypic and morphological subtypes. In ALL patients, response to available
133 therapeutic regimens is markedly variable even in each subtype. Resistant patients may need
134 new therapeutic strategies designed based on underlying mechanisms of the cancers. This aim
135 is feasible with the investigation about molecular mechanisms behind the formation of cancer
136 cells. In this regard, cell cycle regulators are at the focus center. APC/C, as a critical cell

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137 cycle regulator, seems to be important in cancer formation and progression (3). Mutations in
138 the subunits of the APC/C complex have been documented in many types of cancers
139 including breast cancer, colon cancer, glioma, and hepatocarcinoma . Recent studies also
140 have shown increased APC/C subunits/ activator expression in a variety of solid tumors and
141 hematologic malignancies. Other studies demonstrated a significant correlation between
142 APC/C levels of activation and disease prognosis. Our results showed a statically significant
143 increased in the levels of APC2 and APC7 expression in ALL patients, but it was not
144 significantly correlated with immunological subtypes of ALL, chromosomal translocation,
145 FAB classification, gender, blast percent and the age of the patients.

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

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

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

167

168 **Conclusion**

169 The main challenge of dividing cells is duplication of 6 billion bases of DNA and accurate
170 segregation of this DNA content between daughter cells. The fidelity of genome content
171 during cell division is controlled in three major checkpoints. Disruption of these checkpoints
172 is common hallmarks of human cancers. Spindle assembly checkpoint (SAC) is the main
173 regulator of chromosome segregation in metaphase that regulates APC/C activity as an
174 effector molecule (36-43). Overexpression of APC/C may cause decreased inhibition by SAC
175 and subsequently may lead to chromosome missegregation and aneuploidy. Our study
176 demonstrated that APC2 and APC7 are overexpressed simultaneously in newly cases of ALL.
177 Accordingly, with respect to the role of APC/C in chromosomal integrity, it is not unexpected

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178 to see high rate of chromosome aberrancies such as aneuploidy and translocation in ALL
179 leukemic blasts. So this over-activation may be involved in the initiation of malignancy and
180 its evolution. Also APC/C over expression may promotes cell proliferation, a feature related
181 to poor prognosis in ALL patients, so the determination of the rate of APC/C subunits
182 expression may help us to find poor prognosis ALL patients and to better risk-stratify
183 patients beside using the conventional risk factors.

184

185 **Conflict of interest**

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

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189 **References**

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191 1. Pui, C.H. and W.E. Evans, Acute lymphoblastic leukemia. N Engl J Med, 1998.
192 339(9): p. 605-15.
193 2. Pui, C.H., L.L. Robison, and A.T. Look, Acute lymphoblastic leukaemia. Lancet,
194 2008. 371(9617): p. 1030-43.
195 3. Campana, D. and G. Janossy, Proliferation of normal and malignant human immature
196 lymphoid cells. Blood, 1988. 71(5): p. 1201-10.
197 4. Pui, C.H. and W.E. Evans, Treatment of acute lymphoblastic leukemia. N Engl J
198 Med, 2006. 354(2): p. 166-78.
199 5. Bassan, R. and D. Hoelzer, Modern therapy of acute lymphoblastic leukemia. J Clin
200 Oncol, 2011. 29(5): p. 532-43.
201 6. Hunger, S.P. and C.G. Mullighan, Acute Lymphoblastic Leukemia in Children. N
202 Engl J Med, 2015. 373(16): p. 1541-52.
203 7. Inaba, H., M. Greaves, and C.G. Mullighan, Acute lymphoblastic leukaemia. Lancet,
204 2013. 381(9881): p. 1943-55.
205 8. Stock, W., et al., Cell cycle regulatory gene abnormalities are important determinants
206 of leukemogenesis and disease biology in adult acute lymphoblastic leukemia. Blood,
207 2000. 95(7): p. 2364-71.
208 9. Sivakumar, S. and G.J. Gorbsky, Spatiotemporal regulation of the anaphase-
209 promoting complex in mitosis. Nat Rev Mol Cell Biol, 2015. 16(2): p. 82-94.
210 10. Chang, L., et al., Atomic structure of the APC/C and its mechanism of protein
211 ubiquitination. Nature, 2015. 522(7557): p. 450-4.
212 11. Sivaprasad, U., Y.J. Machida, and A. Dutta, APC/C--the master controller of origin
213 licensing? Cell Div, 2007. 2: p. 8.
214 12. Derive, N., et al., Bub3-BubR1-dependent sequestration of Cdc20Fizzy at DNA
215 breaks facilitates the correct segregation of broken chromosomes. J Cell Biol, 2015.
216 211(3): p. 517-32.
217 13. Zhou, Z., et al., Insights into APC/C: from cellular function to diseases and
218 therapeutics. Cell Div, 2016. 11: p. 9.
219 14. Wasch, R. and D. Engelbert, Anaphase-promoting complex-dependent proteolysis of
220 cell cycle regulators and genomic instability of cancer cells. Oncogene, 2005. 24(1):
221 p. 1-10.
222 15. Rahimi, H., et al., The expression pattern of APC2 and APC7 in various cancer cell
223 lines and AML patients. Adv Med Sci, 2015. 60(2): p. 259-63.

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224 16. Wang, L., et al., Targeting Cdc20 as a novel cancer therapeutic strategy. Pharmacol
225 Ther, 2015. 151: p. 141-51.
226 17. Zeng, X., et al., Pharmacologic inhibition of the anaphase-promoting complex induces
227 a spindle checkpoint-dependent mitotic arrest in the absence of spindle damage.
228 Cancer Cell, 2010. 18(4): p. 382-95.
229 18. Huang, H.C., et al., Evidence that mitotic exit is a better cancer therapeutic target than
230 spindle assembly. Cancer Cell, 2009. 16(4): p. 347-58.
231 19. Association, W.M., World Medical Association Declaration of Helsinki: ethical
232 principles for medical research involving human subjects. Jama, 2013. 310(20): p.
233 2191-4.
234 20. Bene, M.C., et al., Proposals for the immunological classification of acute leukemias.
235 European Group for the Immunological Characterization of Leukemias (EGIL).
236 Leukemia, 1995. 9(10): p. 1783-6.
237 21. Chomczynski, P. and N. Sacchi, The single-step method of RNA isolation by acid
238 guanidinium thiocyanate-phenol-chloroform extraction: twenty-something years on.
239 Nat Protoc, 2006. 1(2): p. 581-5.
240 22. Schmittgen, T.D. and K.J. Livak, Analyzing real-time PCR data by the comparative
241 C(T) method. Nat Protoc, 2008. 3(6): p. 1101-8.
242 23. Pui, C.H., et al., Biology, risk stratification, and therapy of pediatric acute leukemias:
243 an update. J Clin Oncol, 2011. 29(5): p. 551-65.
244 24. Yeoh, E.J., et al., Classification, subtype discovery, and prediction of outcome in
245 pediatric acute lymphoblastic leukemia by gene expression profiling. Cancer Cell,
246 2002. 1(2): p. 133-43.
247 25. Bennett, J.M., et al., Proposals for the classification of the acute leukaemias. French-
248 American-British (FAB) co-operative group. Br J Haematol, 1976. 33(4): p. 451-8.
249 26. Ross, M.E., et al., Classification of pediatric acute lymphoblastic leukemia by gene
250 expression profiling. Blood, 2003. 102(8): p. 2951-9.
251 27. Wang, Q., et al., Alterations of anaphase-promoting complex genes in human colon
252 cancer cells. Oncogene, 2003. 22(10): p. 1486-90.
253 28. Lehman, N.L., et al., Oncogenic regulators and substrates of the anaphase promoting
254 complex/cyclosome are frequently overexpressed in malignant tumors. Am J Pathol,
255 2007. 170(5): p. 1793-805.
256 29. Sivakumar S, Gorbsky GJ. Spatiotemporal regulation of the anaphase-promoting
257 complex in mitosis. Nat Rev Mol Cell Biol. 2015;16(2):82-94.
258 30. Sivaprasad U, Machida YJ, Dutta A. APC/C--the master controller of origin
259 licensing? Cell Div. 2007;2:8.
260 31. Kraft, C., et al., Mitotic regulation of the human anaphase-promoting complex by
261 phosphorylation. Embo j, 2003. 22(24): p. 6598-609.
262 32. Kramer, E.R., et al., Mitotic regulation of the APC activator proteins CDC20 and
263 CDH1. Mol Biol Cell, 2000. 11(5): p. 1555-69.
264 33. Pich, A., et al., Prognostic value of the rapidity of bone marrow blast cell proliferation
265 in adult acute lymphoblastic leukemia. Leukemia, 2004. 18(1): p. 172-4.
266 34. Schneider, P., et al., The growth of highly proliferative acute lymphoblastic leukemia
267 may be independent of stroma and/or angiogenesis. Leukemia, 2001. 15(7): p. 1143-
268 5.
269 35. Ness, K.K., et al., Adverse effects of treatment in childhood acute lymphoblastic
270 leukemia: general overview and implications for long-term cardiac health. Expert Rev
271 Hematol, 2011. 4(2): p. 185-97.
272 36. Nasri H, Dehghan Shahreza F. Defensins usage as novel therapeutic and diagnostic
273 approach. Immunopathol Persa. 2015;1(1):e05.

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274 37. Shakweer MM, El-Sheshtawy NM. Emerging role of Treg FOXP3 expression in cancer
275 prognosis and autoimmune diseases. Immunopathol Persa. 2017;3(1):e01.
276 38. Dehghan Shahreza F. From oxidative stress to endothelial cell dysfunction. J Prev
277 Epidemiol. 2016; 1(1):e04.
278 39. Naqvi R. Use of rituximab in immunological disorders. Immunopathol Persa.
279 2016;2(1):e06
280 40. Salami A, Amiri M. On the occasion of world cancer day 2017; breast cancer. J Ischemia
281 Tissue Repair. 2017;1(1):e02.
282 41. Nasri H. Sudden onset of renal failure requiring dialysis associated with large B-cell
283 lymphoma of colon. J Nephropathol. 2012 Oct;1(3):202-6.
284 42. Balwani MR, Kute VB, Shah PR, Wakhare P, Trivedi HL. Secondary renal amyloidosis
285 in a patient of pulmonary tuberculosis and common variable immunodeficiency. J
286 Nephropharmacol. 2015 Jun 21;4(2):69-71.
287 43. Yaghoubi F, Yarmohammadi M, Vasei M. Paraneoplastic proteinuria in papillary renal
288 cell carcinoma; a case report. J Renal Inj Prev. 2016 Aug 3;5(4):207-9.