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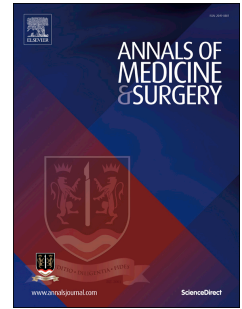
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RELATION BETWEEN EXPRESSION OF hMLH1 AND p53 mRNA GENES IN THE FECES OF PATIENTS WITH COLORECTAL CARCINOMA. Cross-sectional study.

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1 **RELATION BETWEEN EXPRESSION OF hMLH1 AND p53 mRNA GENES, IN THE**
2 **FECES OF PATIENTS WITH COLORECTAL CARCINOMA. Cross-sectional study.**

3
4 **ABSTRACT**

5 Colorectal carcinoma (CRC) is one of the main public health problems. The mortality of
6 CRC is about 8%. Early detection of CRC is very important to prevent death because this cancer
7 could be cured through surgery if the diagnosis can be made as early as possible. Therefore
8 screening strategy for early detection of CRC is critical in reducing mortality. Many investigations
9 supporting the detection of CRC have been developed, including the fecal DNA mutation test using
10 advanced cytological techniques. It is capable of assessing colonocytes for the presence of DNA,
11 RNA, and protein as molecular biomarkers of neoplasia in CRC, including p53 and hMLH1. This
12 study implemented observational approach with a cross-sectional study of the feces of patients
13 with CRC regardless of the stage and grade. The purpose of this study was to determine the
14 expression of the hMLH1 and p53 mRNA genes in the feces of 48 patients with CRC from two
15 hospitals in Indonesia, Siloam Hospitals in Cikarang and Dr. Wahidin Sudirohusodo Hospital in
16 Makassar. The results showed that all adenocarcinoma feces samples with various tumor stages
17 and grades had excess mRNA expression (more than twice the normal amount in Fold Change
18 units) for both the hMLH1 and p53 genes. The average expression of the hMLH1 mRNA gene was
19 the highest at stage two and grade one, while the lowest was at stage four and grade three. In
20 contrast, the average p53 mRNA gene expression was the highest at stage four and grade three,
21 while the lowest was at stage two and grade one. The study suggested that there was a relation
22 between and the expression of hMLH1 and p53 mRNA gene . . We concluded that while both
23 hMLH1 and p53 genes in patients' feces with CRC were overexpressed, they did not significantly
24 affect the grade of CRC.

25
26 **Keywords:** hMLH1, p53, Colorectal Carcinoma

27 Research Registry : researchregistry7319

28 Ethical Approval : 798/UN4.6.4.5.31/PP36/2020 (8 December 2020)

1. Background

Colorectal carcinoma (CRC) is one of the main public health problems and concerns, both in developed and developing countries (1). The frequency of CRC ranks third in both men and women, and the mortality is about 8%. CRC screening strategy plays an important role in preventing death and reducing CRC because this cancer can be cured through surgery if an appropriate diagnosis is taken place as early as possible. There are available methods to investigate CRC including Fecal Occult Blood Tests (FOBT) which are non-invasive and very useful with sigmoidoscopy or colonoscopy (2). However, the tests have relatively high false-positive rate are nonspecific, and not sensitive enough for early-stage CRC detection, even though high sensitivity results were reported in the advanced stage of CRC (2).

A study by Dong *et al.* identified the combination of p53, BAT 26, and K-ras gene mutations in feces in 71% of colorectal carcinoma patients (3). Ahlquist *et al.* improved the detection sensitivity to 91% using a panel consisting of the same genes with an addition of one more gene, APC, to the combination (4). It has also been acknowledged that RNA-based detection methods are more comprehensive than DNA, protein, or methylation-based methods (5, 6).

Three major alteration can occur in CRC, namely microsatellite instability (MSI), chromosomal instability (CIN), and CpG island methylator phenotype (CIMP) which modify the DNA, RNA, protein, or metabolites, which can therefore be used as a biomarker for CRC detection in tumor samples and blood or feces, . The most widely used biomarkers for CRC at present are the presence of MSI and KRAS mutations in tumor samples which are used to classify tumors, make disease prognosis and administer a therapy. Although other biomarkers, including determination of FOBT and CEA, have also been used for making disease prognosis, they tend to have high specificity but very low sensitivity (7, 8).

On the other hand, MLH1 gene is known as the mismatch repair (MMR) gene that provides instructions for making proteins that are crucial in DNA repair. The MLH1 protein in combination with another protein, PMS2 (produced from the PMS2 gene), form a two-protein complex called a dimer. There are seven MMR DNA proteins in humans namely MLH1, MLH3, MSH2, MSH3, MSH6, PMS1, and PMS2 (9).

Another gene, TP53, provides instructions for making tumor suppressor proteins. p53 functions not only as a negative regulator of the cell cycle but also in the cell's response to DNA damage or stress. Cellular p53 protein levels and function are regulated by another gene product namely the mouse double minute 2 oncogene (MDM2) as a negative regulator. MDM2 is a transcriptional target of p53, and expression is induced by the binding of p53 to an internal promoter in the MDM2 gene that can cause mutations (10).

Most TP53 mutations change a single amino acid in the p53 protein that lead to the production of an altered protein version that can no longer control cell proliferation and cannot induce cell apoptosis in cells accumulated with mutated or damaged DNA. As the consequences, tumor cells grow following uncontrolled cell division.

In this case, Gene Mismatch Repair (MMR) plays an important role in the recognition and error repair after replication to prepare for cell division. The errors accumulation in repeated DNA fragments lead to mutations in the target gene. Mutations in the mismatch DNA repair gene, i.e., defective MMR DNA system (microsatellite instability) have been reported to be about 20% in the sporadic CRC and Lynch syndrome. Methylation by CpG island methylator (CIMP), a dinucleotide methylation, takes place at the initial transcriptional site upstream of many genes. They are reported to be associated with approximately 15% to 20% of sporadic CRCs. The CpG

74 island methylator phenotype mainly carries out hypermethylation of the promoter region of any
75 genes. These methylators methylate certain marker genes in the tumor positives.

76 It is currently understood that MSI and CIN tumors develop through two different pathways
77 in the colon, the sessile serrated pathway induces MSI tumors in the proximal colon, and the
78 traditional adenoma-carcinoma pathway induces CIN tumors in the distal colon. While APC is
79 thought to be the precursor to CIN tumor, BRAF is one of the earliest identified alterations among
80 MSI precursors. During CIN tumorigenesis, genetic complexity intensifies due to chromosomal
81 instability, while on the other hand, CIMP leads to epigenetic instability among MSI precursors,
82 ultimately affecting MLH1, as a cause of MSI.

83 A deficiency in MMR generates an environment in which cells rapidly build up mutations,
84 including those that induce cancer development. The MMR core in humans consists of three
85 heterodimeric protein complexes involved in recognizing MMR. The first protein complex,
86 hMUTS α (consisting of hMSH2 and hMSH6) recognize and preferentially bind single-nucleotide
87 mismatches, while the second, hMUTS β (hMSH2 and hMSH3) recognize and preferentially bind
88 insertion-deletion loops. After mismatch recognition, the third protein complex, hMUTL,
89 consisting of hMLH1 paired with hPMS2 or hPMS1, is recruited into the lesion where ATPase and
90 enzymatic endonuclease activity are engaged to complete the repair process. TP53 mutations in
91 CRC were found to be less common in MSI tumors (10–20% of cases) than in CIN tumors (~50–
92 60% of cases) which suggested that MMR deficiency does not cause TP53 mutations in MSI
93 tumorigenesis. Then loss of heterozygosity (LOH) can occur if the wild-type allele remains or the
94 short arm deletion of chromosome 17 is present. In contrast, copy-neutral LOH (cnLOH) occurs
95 when there is no net change in the copy number of the affected allele (11).

96 MSI has been used as a clinical tool to diagnose tumor. Microsatellite repeats specific for
97 MSI can be detected by a relatively simple PCR amplification. MSI can also be detected by
98 comparing the length of nucleotide repeats in tumor cells and adjacent normal cells using
99 fluorescent primers in RT-PCR (Real Time Polymerase Chain reaction) coupled with capillary
100 electrophoresis which was the approach in this study. PCR is a commonly used detection method,
101 using primers that amplify target DNA fragments *in vitro* (12). This study was aimed at
102 determining the expression of hMLH1 and p53 mRNA genes in the feces of patients with colorectal
103 carcinoma in various stages and grades of tumors using RT-PCR. This study is an observational
104 study with a cross-sectional approach on the feces of patients with colorectal carcinoma regardless
105 of the stage of the disease.

106

107 2. Materials and Methods

108 Our work was fully compliant with the STROCSS criteria and has been reported with the
109 checklist completed (13). Also, our work has been registered with a unique identifying number
110 (UIN): Researchregistry7319 ([https://www.researchregistry.com/browse-the-
111 registry#home/registrationdetails/617ed3cb0ec644001e8e6300/](https://www.researchregistry.com/browse-the-registry#home/registrationdetails/617ed3cb0ec644001e8e6300/)).

112 Ethical approval has been approved by ethics commission of Faculty of Medicine,
113 Hasanuddin University reference no. 798/UN4.6.4.5.31/PP36/2020.

114 2.1. Diagnosis and determination of tumor staging

115 The diagnosis of carcinoma was preliminary established based on histopathological
116 examination of the tumor mass sample through endoscopy or surgery.

117 Tumor staging was determined by the results of intraoperative findings, including tumor
118 extension, lymph node involvement, and metastasis. The tumor mass substrate was adjusted
119 according to the topography of the colon and rectum. The research material was taken through the

120 Digital Rectal Examination (DRE) or directly from the patients' feces.

121 The inclusion criteria of research subjects to maintain objectivity of the samples were CRC
122 patients based on histopathological examination results as adenocarcinoma in various stages and
123 grades. Exclusion criteria for research subjects were CRC patients who were on neo-adjuvant
124 chemotherapy and/or radiotherapy, which could affect the expression of the hMLH1 and p53
125 genes. The CRC stage was determined by the TNM system and histopathological staging. CRC
126 was graded based on the histopathological examination results.

127 2.2. Selection of samples and RT-PCR technique

128 The sample was an affordable population that meets the research criteria and expresses their
129 willingness to participate in the study in writing by signing the consent form to participate in the
130 study. Samples was taken from CRC patients in Siloam Hospitals and Dr. Wahidin Sudirohusodo
131 hospital located in Cikarang, Bekasi and Makassar, Indonesia, respectively. Samples were
132 collected from December 2020 to May 2021.

133 Adequate sample size according to the Harry King nomogram is 41, however in order to
134 anticipate possible dropout of respondents, an extra 10% was added making a total of 48
135 respondents. We did not categorized the samples in two groups. No division of intervention
136 between groups was applied. All samples were treated as one.

137 Feces were sampled from patients with CRC using the DRE method or directly from the
138 patients' feces. We used 100 $\mu\text{g}/\mu\text{l}$ feces per sample. Initially, debris and colonocytes in the feces were
139 separated by centrifuging at 1000rpm for two min. Then DNA extraction was performed. Real-time PCR
140 was then carried out to identify the mRNA expression profile of the two target genes, namely hMLH1 and
141 p53.

142

143 2.3. Data analysis

144 The data obtained were analyzed by using the hMLH1 and p53 mRNA gene expression test
145 according to tumor stage and grading, which consisted of data homogeneity test (Levene statistic),
146 data normality test using the Shapiro-Wilk test, and continued with the One-way ANOVA test
147 followed by with LSD Test to group non-significantly different samples.

148

149 3. Results

150 The topographic features of the CRC substrates were 35% the rectum, followed by 31% the
151 sigmoid colon, 21% ascending colon, 6% transverse colon, and 6% descending colon. Most of the
152 tumor were of stage three tumor (35%), followed by stage 2 (33%) and stage 4 tumors (31%).
153 Stage 1 tumor was not found in the samples.

154 The tumor grade was dominated by Well Differentiation grade 1 (60%), then Moderate
155 Differentiation grade 2 (27%), and Poorly Differentiation grade 3 (13%). All adenocarcinoma fecal
156 samples showed overexpression of mRNA (more than twice the normal amount in Fold Change
157 units) of hMLH1 and p53 genes in various stages (stages 2,3,4) and tumor grades (G1, G2, G3).
158 The mRNA expression of the hMLH1 gene with the highest average value was in grade 1 (Mean:
159 8.66), the lowest was grade 3 (Mean: 7.88), the mRNA expression of the p53 gene with the highest
160 average value was in grade 3 (Mean: 10.32) and the lowest was grade 1 (Mean: 9.22) (Table 1,
161 Fig.1

162

163 The mRNA expression of the hMLH1 gene with the highest average value was at stage 2
164 (Mean: 9.59875), the lowest was stage 4 (Mean: 7.19440), the mRNA expression of the p53 gene
165 with the highest average value was at stage 4 (Mean: 11.68887) and the lowest was stage 2 (Mean:
166 7.40544) (Table 2., Fig.2.).
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168 The mRNA expression data for the hMLH1 and p53 genes respectively had values of 0.121 and
169 0.289 (>0.05), which means all data were homogeneous. The Shapiro-Wilk test showed that the
170 data were normally distributed ($\text{sig} > 0.05$). The results of the one-way Anova test showed a
171 significant difference in the mRNA expression data for the hMLH1 and p53 genes ($\text{sig.} < 0.05$).
172 The LSD test showed significant differences in the mRNA expression values of the hMLH1 and
173 p53 genes between all different stages of colorectal cancer.

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174 The mRNA expression data for the hMLH1 and p53 genes had values > 0.05 , meaning that
175 all data were homogeneous. The data were normally distributed (sig > 0.05). The results of the one-
176 way Anova test showed that there was no significant difference in the expression data for hMLH1
177 and p53 (sig. >0.05), further LSD test was not performed (Fig.1.).

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178 4. Discussion

179 This study suggested that the RT-PCR is highly sensitive and specific for detecting CRC at
180 an early stage, allowing patients to be treated before they reach more severe or metastatic disease
181 stages. Preventive screening of at-risk populations is possible using this relatively rapid technique,
182 which can reduce mortality and morbidity rates of CRC (12).

183 The results of statistical analysis showed significant differences in the mRNA expression
184 values of the hMLH1 and p53 genes among all different CRC stages. Although the results obtained
185 were significantly different, the mean value of p53 gene mRNA expression was not directly
186 proportional to the stage it affected. At stage two, the mRNA expression of the p53 gene was lower
187 than that of stage four. This was different from the mean value of mRNA expression of the hMLH1
188 gene which was directly proportional to the stage, where the mRNA expression value of the
189 hMLH1 gene was higher in stage two compared to the lower expression value in stage four.
190 However, the CRC grade for the mRNA expression of the hMLH1 and p53 genes showed no
191 significant difference.

192 Tumor suppressor gene which is referred as "tumor suppressor gene", or by its synonym
193 "anti-oncogene", or "recessive oncogene" is a term used to describe genes that can inhibit tumor
194 growth. The hMLH1 and p53 genes are genes that belong to this group. Multistep carcinogenesis
195 requires the action of activated oncogenes and the inactivation of tumor suppressor genes
196 (14). Both the tumor suppressor and onco genes are believed to play a critical role in the initiation
197 and development of most neoplasms, including CRC. The expression of p53 mRNA gene in our
198 study contradicted a tumor suppressor gene pattern but coincided with that of oncogene. We
199 speculated that this change of pattern was likely due to mutation, as this phenomenon has been
200 observed by other researchers in tumor cells. Changes in p53 expression have been described in
201 between 44% (15) and 55% of colon tumors. p53 gene mutations in two tumors associated with
202 increased mRNA expression have been reported (16). Remvikos *et al.* (1990) found significant
203 associations between the increase of p53 and the presence of DNA aneuploidy (17). In rectal
204 carcinoma, p53 mutation and loss of the allele on the short arm of chromosome 17 appears to cause
205 genetic changes that deactivate the function of gene p53 as the tumor suppressor, that the changes
206 in this gene cause the p53 protein to malfunction (18).

207 Previously p53 was reported in many nonviral altered tumor cells, but not in normal cells,
208 and is therefore called a cellular tumor antigen. However, with better detection methods, p53 was
209 also found in normal cells, although in very small levels. Research by Mercer *et al.* showed that
210 p53 expression is required for resting cell transit from G0 to G1 (14). Transfection of p53 into
211 primary cells led to the immortalization of these cells, while cotransfection of p53 with activated
212 ras oncogene caused the conversion of cells to completely alter the phenotype. Furthermore,
213 transfection vector of p53 expression into various cells suggested that the effect of overexpressed
214 p53 was related to cell transformation or tumorigenesis (14). The overexpression of p53 in tumor
215 cells was most often due to the metabolic stabilization of the p53 protein at the post-translational
216 level. This leads to transformed cells containing an approximately 100-fold increase in p53. An
217 increase in the amount of p53 in tumor cells will propel these cells from one cycle of the cell cycle
218 to the next, which will lead to increased proliferation.

219 P53 mutations are the most frequently detected genetic changes in human cancers found in
220 more than 50% of all human cancers. The p53 gene mutation alters the conformation of the nuclear
221 protein product, which can inactivate the wild-type p53 protein. Elevated p53 protein expression
222 or inactivation mutations of the p53 gene have been demonstrated in various malignant tumors in
223 humans including carcinomas of the colon and rectum, breast, prostate, lung, stomach, thyroid,

224 and liver. In colorectal carcinoma, the occurrence of p53 mutations varies and is found in 50% to
225 70% of cases.

226 Based on the histological assessment of colorectal adenocarcinoma, the results of the study
227 conducted by Khudier et al showed that 41.2% were well-differentiated, 47.1% were moderately
228 differentiated, and 11.8% were poorly differentiated. While the study was conducted by Valera et
229 al showed that 57.6% were well-differentiated, 39.6% were moderately differentiated, and 2.8%
230 were poorly differentiated.

231 In their study, there was no relationship between CRC stage and p53 expression which was
232 in line with the results of research conducted by Demirbas and colleagues who also did not find a
233 significant relationship between p53 and stage but did not agree with the results of the research
234 conducted by Jackson and colleagues who found a significant relationship between p53 and CRC
235 stage (19).

236 Evidence of p53 mutations is found in most CRCs in the clinical population indicating that
237 chromosomal instability is responsible for the majority of colorectal carcinomas. No association
238 could be found between p53 expression and histologic type, histological grade, and stage (19).

239 The MLH1 gene provides instructions for making proteins that play an important role in
240 repairing errors made during DNA replication in preparation for cell division. The MLH1 protein
241 combines with another protein called PMS2 to form a protein complex. Repair is done by removing
242 the part of the DNA that contains errors and replacing the part with the corrected DNA sequence.
243 The MLH1 gene is a member of a set of mismatch repair (MMR) genes. MMR deficiency is
244 associated with hereditary non-polyposis colorectal cancer (Lynch syndrome) and sporadic cases
245 of CRC. This MMR system is well known for maintaining the overall stability of the genetic
246 material, deficient cells exhibit a mutator phenotype with a high rate of microsatellite mutations.

247

248 **5. Conclusions**

249 This study showed that there was a relation between the hMLH1 mRNA gene expression
250 and the stage of CRC, the higher the value of fold change expression, the lower the stage of CRC.
251 On the other hand, negative correlation was shown between the p53 mRNA gene expression and
252 the CRC stages, the higher the value of fold change expression, the higher the stage of CRC. It
253 was concluded that the hMLH1 and p53 genes in respondents' feces with CRC were
254 overexpressed, but the expression value did not suggest the stages of CRC. hMLH1 mRNA gene
255 expression is in accordance with its function, however p53 gene had an altered expression pattern
256 which might be due to mutation.

257

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264

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267

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Table 1. Correlation between mRNA expression of the hMLH1 and p53 genes and preliminary grading results

		N	mean	Std Deviation	Std. Error	95% Confidence Interval for mean		min	max
						Lower Bound	Upper Bound		
Expression mRNA	Grade1	29	8.66369	1.213515	0.225344	8.20209	9.12529	6,399	10,607
	Grade2	13	8.23815	1.435497	0.398135	7.37069	9.10562	6.168	10.282
hMLH1	Grade3	6	7.88483	1.522059	0.621378	6.28753	9.48214	6.411	10,607
Total		48	8.45108	1.315768	0.189915	8.06902	8.83314	6.168	10,607
Expression mRNA.	Grade1	29	9.21800	2.286227	0.424542	8.34837	10.08763	5,720	13,880
	Grade2	13	10.12615	2.239878	0.621230	8.77261	11.47970	6.268	13.013
p53.	Grade3	6	10.32367	2.820682	1.151539	7.36354	13.28379	5.138	13,349
Total		48	9.60217	2.339803	0.337721	8.92276	10.28157	5.138	13,880

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Table 2. Correlation between mRNA expression of the hMLH1 and p53 genes and preliminary staging results

mRNA expressio n Gene	Stage	N	mean	Std. Deviation	Std. Error	95% Confidence Intervals for Mean		min	max
						Lower Bound	Upper Bound		
hMLH1	2	16	9.59875	0.551687	0.137922	9.30478	9.89272	8,663	10.362
	3	17	8.48124	1.236147	0.299810	7.84567	9.11680	6.168	10,607
	4	15	7.19440	0.737266	0.190361	6.78612	7.60268	6.218	8,779
	Total	48	8.45160	1.316044	0.189955	8.06946	8.83374	6.168	10,607
p53	2	16	7.40544	1.118007	0.279502	6.80969	8.00118	6.051	9.376
	3	17	9.82847	2.059278	0.499448	8.76969	10.88725	5.138	12.656
	4	15	11.68887	1.396820	0.360657	10.91533	12.46240	8,562	13,880
	Total	48	9.60217	2.339803	0.337721	8.92276	10.28157	5.138	13,880

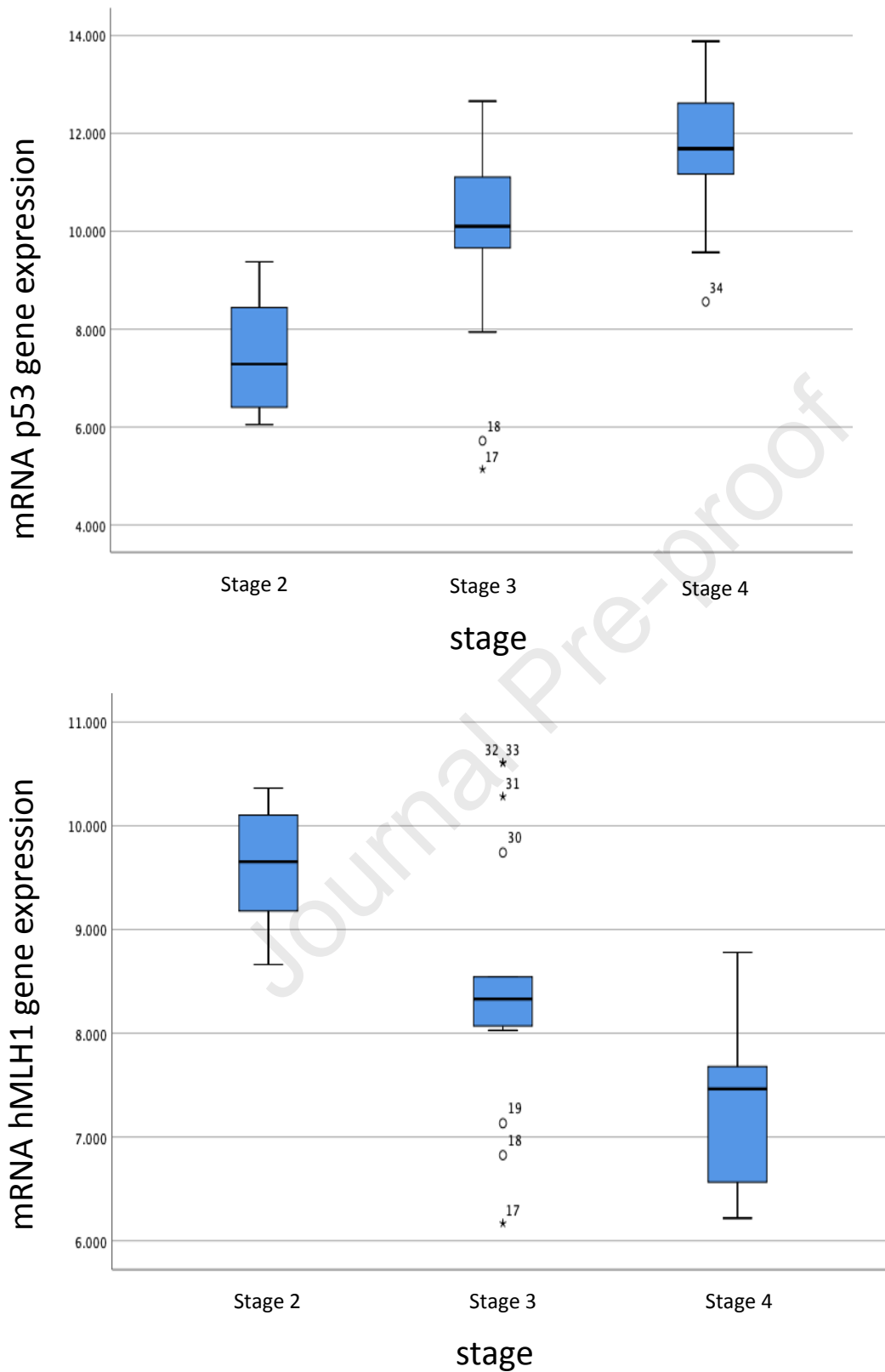


Fig.1. hMLH1 and p53 mRNA gene variables as measured by tumor stage

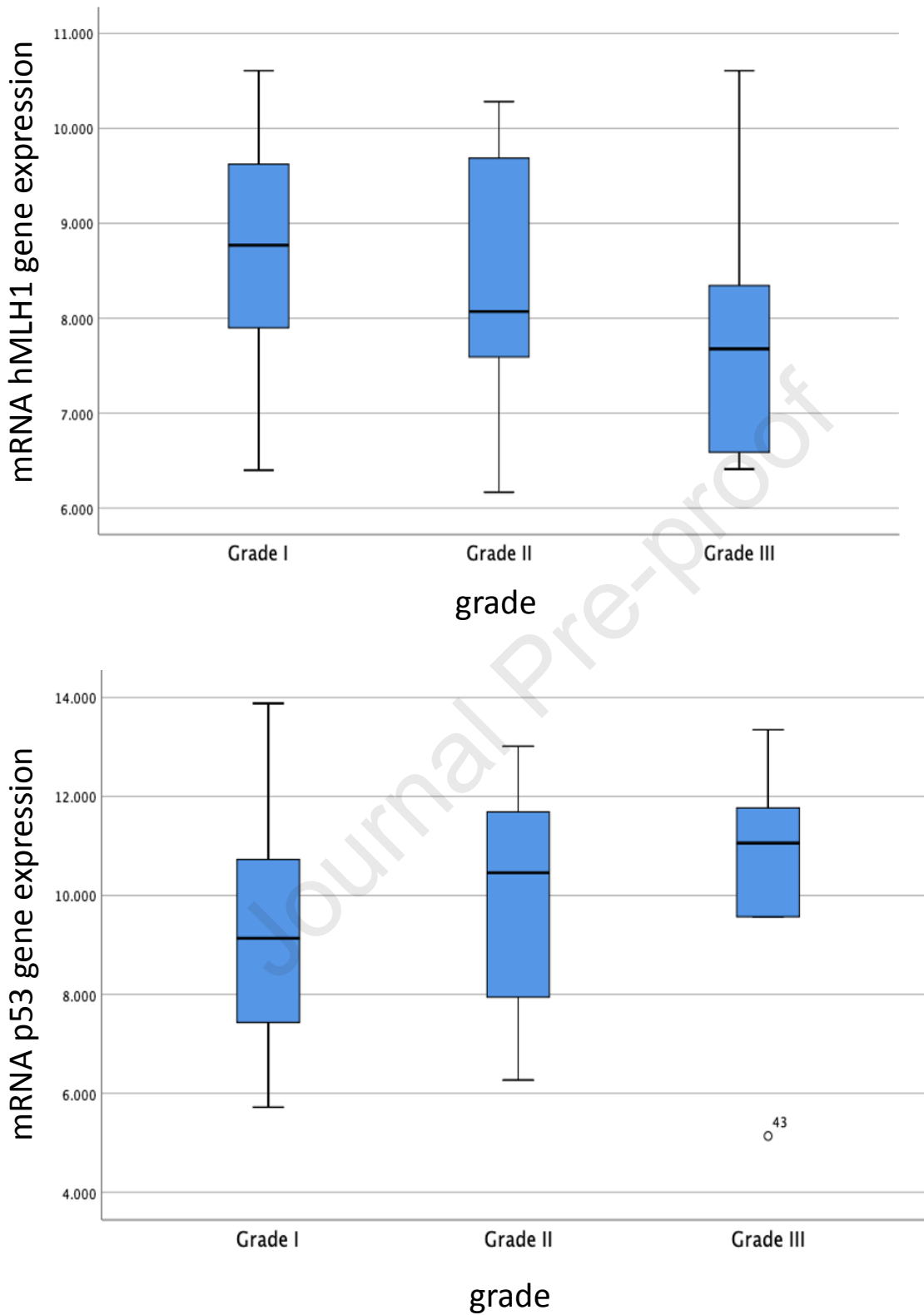


Fig.2. hMLH1 and p53 mRNA gene variables as measured by tumor grade

Highlight :

1. RELATION OF mRNA EXPRESSION OF hMLH1 and p53 GENE IN COLORECTAL CARCINOMA PATIENTS FECES
2. mRNA EXPRESSION IN FECES COLORECTAL CARCINOMA BY RT-PCR EXAMINATION
3. GENE hMLH1 and p53 IN FECES COLORECTAL CARCINOMA PATIENTS

Journal Pre-proof

Annals of Medicine and Surgery

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The study was funded privately by authors.
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Ethical Approval

Research studies involving patients require ethical approval. Please state whether approval has been given, name the relevant ethics committee and the state the reference number for their judgement.

Ethical approval has been approve by ethics commission of Faculty of Medicine, Hasanuddin University reference no. 798/UN4.6.4.5.31/PP36/2020

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Author contribution

Please specify the contribution of each author to the paper, e.g. study concept or design, data collection, data analysis or interpretation, writing the paper, others, who have contributed in other ways should be listed as contributors.

Ryanto Karobuana Sitepu (RKS), Rosdiana Natzir (RN), Warsinggih, (WS), Mochammad Hatta (MH) initiated and designed the study, drafted the manuscript.
All authors have read and approved the final manuscript.

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