

## Prognostic Significance of Nuclear $\beta$ -Catenin Expression in Patients with Colorectal Cancer from Iran

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**Background:** Beta catenin plays a key role in cancer tumorigenesis. However, its prognostic significance in patients with colorectal cancer (CRC) remains controversial. It has been demonstrated that 90% of all tumors have a mutation in individual components of multiple oncogenes in *Wnt*/ $\beta$ -catenin pathway. Accumulation of nuclear  $\beta$ -catenin in cytoplasm leads to uncontrolled cell proliferation. Thus, nuclear  $\beta$ -catenin accumulation may be a valuable biomarker associated with invasion, metastasis and poor prognosis of CRC.

**Objectives:** In this study the prognostic value of beta catenin expression in 165 Iranian CRC patients was evaluated.

**Patients and Methods:** In this cross sectional retrospective study immunohistochemistry analyses of formalin-fixed paraffin-embedded (FFPE) tumor tissues were performed to characterize the expression of nuclear  $\beta$ -catenin in a series of 165 Iranian patients with colorectal carcinoma. Heat-induced antigen retrieval using the microwave method was applied for all staining procedures. Staining was scored independently by two observers, and a high level of concordance (90%) was achieved. Statistical analysis was done using the SPSS software for Windows, version 13.0.0 (SPSS Inc., Chicago, IL). Two-tailed  $P < 0.05$  was considered statistically significant.

**Results:** The patients consisted of 85 males and 80 females. Eighty-eight patients had primary tumor of the rectum and sigmoid, while 77 patients had primary tumor of the colon. The mean period of follow-up was  $47.2 \pm 10$  months and the median period of follow-up was 38 months (range 6 - 58) for each patient. Of 165 tumors, 32 tumors (19.39%) showed expression of  $\beta$ -catenin and 133 (80.6%) were negative for  $\beta$ -catenin expression. Based on our findings the distribution of Microsatellite Instability (MSI) status differed between patients with nuclear  $\beta$ -catenin positive and negative tumors and this difference was significant ( $P = 0.001$ ). Patients with nuclear  $\beta$ -catenin positive expression profile were found to be younger than patients with negative nuclear  $\beta$ -catenin expression ( $P = 0.010$ ). Univariate and multivariate analysis showed that tumors with  $\beta$ -catenin expression had a poorer prognosis compared to tumors without  $\beta$ -catenin expression.

**Conclusions:** According to our findings, the distribution of nuclear  $\beta$ -catenin expression is a poor prognostic marker in patients with colon cancer.

**Keywords:** Beta Catenin; Colorectal Cancer; Prognostic Biomarkers; Neoplasm Staging

### 1. Background

Colorectal cancer (CRC) is a widespread malignancy in most countries, with a high incidence in Asian countries (1). In the Iranian population, approximately 1130 CRC-related deaths were registered in 2006 (2). Several studies have evaluated the burden of CRC in its sporadic and familial types in the Iranian population (3-5). Transformation of normal colonic mucosa to invasive cancer requires both accumulations of genetic and epigenetic alterations (6).

These alterations in CRC have a distinct molecular pathway: chromosomal instability (CIN), microsatellite instability (MSI) and CpG Island methylator phenotype (CIMP) (6). In the CIN pathway, karyotypic abnormalities,

aneuploidy, loss of heterozygosity and certain mutations are important events (7). Throughout the CIN pathway, several tumor suppressor genes and oncogenes are mutated. Among them, adenomatous polyposis coli (APC),  $\beta$ -catenin, Axin and *GSK3B* genes, which are the members of the *Wnt* signaling pathway, play an important role in colorectal cancer tumorigenesis (8, 9). In CRC, 90% of all tumors have a mutation in individual components of multiple oncogenes in *Wnt*/ $\beta$ -catenin pathway (9). Furthermore,  $\beta$ -catenin is a 92-kDa cellular protein, identified as a protein associated with E-cadherin in maintaining cellular adhesion (10). Also,  $\beta$ -catenin has an independent role in the *Wnt* signal transduction pathway. Mutation in

components of the APC complex leads to increased level of cytoplasmic  $\beta$ -catenin and its translocation to the nucleus (11). Accumulation of nuclear  $\beta$ -catenin in the cytoplasm acts as a transcription factor and binds to T-Cell Factor/Lymphoid Enhancer Factor (TCF/LEF), which leads to activation of target genes including: *CyclinD1*, *c-Myc*, *CD44* and *Survivin*, and at the end it results in uncontrolled cell proliferation in tumor cells (12).

Thus, nuclear  $\beta$ -catenin accumulation may be a valuable biomarker associated with invasion, metastasis and poor prognosis of CRC (13).

## 2. Objectives

The aim of this study was to evaluate nuclear  $\beta$ -catenin expression as a predictor of clinical outcome in Iranian patients with CRC.

## 3. Patients and Methods

### 3.1. Patient Samples and Tumor Data

This retrospective cross sectional study was performed on 165 randomly selected Iranian patients with primary colorectal cancer, who had undergone surgical resection of adenocarcinoma and been referred to the gastroenterology and liver diseases research center of Shahid Beheshti university of medical sciences, Tehran, Iran from 2005 until 2010.

The inclusion criteria consisted of a pathological diagnosis of primary CRC and having undergone a surgical operation for treatment and availability of the patient's formalin-fixed paraffin-embedded sample. On the other hand, patients with Familial Adenomatous Polyposis coli (FAP) and patients without formalin-fixed paraffin-embedded (FFPE) were excluded from the study.

Informed consent was obtained from all patients, or their relatives. Demographic and clinical information was registered prospectively and recorded in a database; this information included age, sex, personal and family medical history, tumor location, tumor, lymph nodes, and metastasis (TNM) stage, tumor differentiation and MSI status. Tumor tissue specimens obtained from the resected tumor were embedded in paraffin blocks according to standard procedures. Microsatellite instability status in tumors was classified as microsatellite instability high (MSI-H), Microsatellite instability low (MSI-L) and MS-L. The TNM staging system was applied to determine the severity of disease and the local or distant extent of disease spread. The TNM staging system of the American joint committee on cancer (AJCC) is the preferred and standard staging system for CRC. Approval of the study was obtained from the regional ethics committee on 7th of July 2004 with code number 681.

### 3.2. Immunohistochemical Procedures

Formalin-fixed, paraffin-embedded blocks with tumor

tissue were sectioned at a nominal size (3 to 5 micrometers thick). Heat-induced antigen retrieval using the microwave method was applied for all staining procedures. In details, the blocks were deparaffinized and processed as follows: 1) the samples were preserved in an oven at 37°C for 24 hours, 2) rinsed with 100% xylol, 100%, 85% and 75% ethanol, and distilled water, 3) exposed to 10% H<sub>2</sub>O<sub>2</sub>, and methanol at a ratio of 1 : 9 for 15 minutes and then rinsed with deionized water, 4) placed in citrate buffered solution (pH = 6) for 24 minutes in a microwave with 800 W and then rinsed with tris-buffered saline (TBS), 5) blocking serum was added to the slides for 15 minutes, and then dried, 6)  $\beta$ -catenin antibodies (monoclonal mouse anti-human  $\beta$ -catenin Dako M3539) were added followed by 45 minutes of incubation at room temperature, and then rinsed with TBS, 7) Envision + visualization system (Dako) was added, followed by 30 minutes of incubation, 8) DAB was added, followed by 10 minutes of incubation, 9) the final samples were rinsed with water, dehydrated in alcohol, and counterstained with hematoxylin. The slides were evaluated by light microscopy. Staining was scored independently by two observers and a high level of concordance (90%) was achieved. All slides were independently reviewed twice and intra-observer disagreements (< 10%) were reviewed a third time followed by a conclusive judgment. Evaluation of nuclear  $\beta$ -catenin expression was performed using a quantitative scale. Nuclear  $\beta$ -catenin staining in the tumor cells was categorized as either positive or negative, whereas the intensity of staining was not considered. The entire tissue sections for each tumor were scanned to estimate the mean value of  $\beta$ -catenin positive nuclei using a two-graded scale (negative, < 5%); (positive, > 5%). Nuclear  $\beta$ -catenin staining was evaluated on whole standard tissue sections from the colon carcinomas. Staining was assessed considering the front of tumor invasion (tumor margin) and the tumor center.

### 3.3. Statistical Analysis

Statistical analysis was performed using the SPSS software program for Windows, version 13.0.0 (SPSS Inc., Chicago, IL). Comparison of variables was performed using Pearson's chi-square test, Fisher's exact test, or the Mann-Whitney U test, depending on the nature of the data. Two-tailed  $P < 0.05$  was considered statistically significant.

Relationships among the clinicopathological factors and  $\beta$ -catenin were analyzed using the chi-square test. For survival analyses, the following variables were assessed: age, sex, location of the tumor (colon versus rectum), tumor-node-metastasis stage, and grade of differentiation (well / moderate versus poor), use of chemotherapy, age at diagnosis, family history, and MSI. Overall survival analyses were done through a Cox proportional hazard function for both univariate and multivariate analyses, and Kaplan-Meier (log-rank test) curves were plotted. Significance for all statistics were recorded if  $P < 0.05$ .

Overall survival was defined as the time from histopathological diagnosis to death from any cause. Patients were followed up until July 2011. Patients who died due to reasons unrelated to colorectal cancer were censored at the time of death and were excluded from the analysis.

#### 4. Results

The patients consisted of 85 males and 80 females. Eighty-eight patients had primary tumor of the rectum

and sigmoid, and 77 patients had primary tumor of the colon (ascending, transverse and descending). The mean period of follow-up was  $47.2 \pm 10$  months for each patient and the median period of follow-up was 38 months.

In the present study 32 out of 165 tumors (19.39 %) expressed nuclear  $\beta$ -catenin and 133 (80.6 %) were negative for nuclear  $\beta$ -catenin expression. The clinicopathological differences between nuclear  $\beta$ -catenin negative and nuclear  $\beta$ -catenin positive tumors are shown in Table 1.

**Table 1.** Demographic and Clinicopathological Features of the Colorectal Cancer Patients <sup>a,b</sup>

Variables	Total	Positive <sup>c</sup>	Negative <sup>c</sup>	P Value
<b>Patients</b>	165	32 (19.4)	133 (80.6)	
<b>Mean age</b>		49.25	56.24	0.010
<b>Gender</b>				0.077
Male	85 (51.5)	12 (37.5)	73 (54.9)	
Female	80 (48.5)	20 (62.5)	60 (62.5)	
<b>Location of tumor</b>				0.024
Ascending colon	24 (14.5)	10 (31.3)	14 (10.5)	
Transverse colon	26 (15.8)	2 (6.3)	24 (18.0)	
Descending colon	27 (16.4)	4 (12.5)	23 (17.3)	
Sigmoid	25 (15.2)	3 (9.4)	22 (16.5)	
Rectum	63 (38.2)	13 (40.6)	50 (37.6)	
<b>Differentiation</b>				0.122
Poorly	18 (10.9)	1 (3.1)	17 (12.8)	
Moderately	46 (27.9)	7 (21.9)	39 (29.3)	
Well	77 (46.7)	16 (50.0)	61 (45.9)	
Unknown	24 (14.5)	8 (25.0)	16 (12.0)	
<b>TNM stage</b>				0.452
I	13 (7.9)	1 (3.1)	12 (9.0)	
II	75 (45.5)	13 (40.6)	62 (46.6)	
III	60 (36.4)	13 (40.6)	47 (35.3)	
IV	17 (10.3)	5 (15.6)	12 (9.0)	
<b>Family history</b>				0.402
No	123 (74.5)	22 (68.8)	101 (75.9)	
Yes	42 (25.5)	10 (31.3)	32 (24.1)	
<b>Vital status</b>				0.008
Living	135 (81.8)	21 (65.6)	114 (85.7)	
Deceased	30 (18.2)	11 (34.4)	19 (14.3)	
<b>Age of diagnosis</b>				0.004
< 50	81 (49.1)	23 (71.9)	58 (43.6)	
> 50	84 (50.9)	9 (28.1)	75 (56.4)	
<b>MSI status</b>				0.001
MSS	102 (61.8)	18 (56.3)	84 (63.2)	
MSL	38 (23.0)	3 (9.4)	35 (26.3)	
MSH	25 (15.2)	11 (34.4)	14 (10.5)	

<sup>a</sup> Abbreviations: MSS, Microsatellite stable; MSI-L, Microsatellite instability low; MSI-H, Microsatellite instability High.

<sup>b</sup> Data are presented as N (%).

<sup>c</sup> Positive/negative expression of nuclear  $\beta$ -catenin.

Patients in the nuclear  $\beta$ -catenin positive group were found to be younger than patients in the nuclear  $\beta$ -catenin negative group (mean age of 49.25 and 56.24 years, respectively), and interestingly this difference was statistically significant ( $P = 0.010$ ).

We also found that the nuclear  $\beta$ -catenin positive group with rectum tumors had a younger age of diagnosis ( $P = 0.004$ ) than the nuclear  $\beta$ -catenin negative group. We observed that the tumors in patients with positive  $\beta$ -catenin expression were predominantly located in the rectum.

In this study, distribution of MSI status differed between patients with nuclear  $\beta$ -catenin positive and negative tumors, and the difference was significant ( $P = 0.001$ ).

The expression level of  $\beta$ -catenin was 56.3% in MSS, 9.4% in MSI-L and 34.4% in MSI-H tumors.

We found no significant differences between patients with nuclear  $\beta$ -catenin positive and negative tumors regarding gender, TNM staging and family history, however

stage II and III were more frequent in  $\beta$ -catenin positive CRCs in comparison with  $\beta$ -catenin negative tumors, yet this difference was not statically significant.

All the characteristics with prognostic value in overall survival were entered in the cox model (univariate and multivariate analysis) including age at diagnostic, tumor stage, MSI status, family history and  $\beta$ -catenin expression. We did not find any significant correlation between overall survival and the aforementioned prognostic factors, except for  $\beta$ -catenin status.

Univariate and multivariate analysis showed that tumors with nuclear  $\beta$ -catenin expression have a poor prognosis compared with tumors without nuclear  $\beta$ -catenin expression (Table 2).

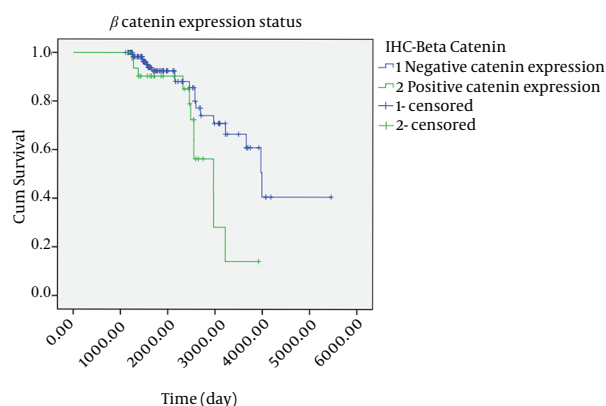
Overall survival curves relative to  $\beta$ -catenin expression were obtained for all colorectal cancer patients. Tumors with nuclear  $\beta$ -catenin expression had a poorer prognosis compared to tumors with negative expression for nuclear  $\beta$ -catenin ( $P = 0.012$ ; Figure 1).

**Table 2.** Univariate and Multivariate Cox Regression Analysis of Possible Prognostic Variables and Parameters That Correlate With Overall Survival

Variables	Univariate Analysis		Multivariate Analysis	
	Hazard Ratio for Death <sup>a</sup>	P Value	Hazard Ratio for Death <sup>a</sup>	P Value
<b>Gender</b>				
Female	1 ref.		1 ref.	
Male	1.356 (0.659 - 2.792)	0.408	0.628 (0.267 - 1.476)	0.286
<b>Location of tumor</b>				
Rectum	1 ref.		1 ref.	
Ascending colon	1.482 (0.517 - 4.251)	0.464	1.347 (0.381 - 4.754)	0.644
Transverse colon	0.532 (0.174 - 1.626)	0.268	0.547 (0.157 - 1.908)	0.344
Descending colon	0.363 (0.103 - 1.278)	0.115	0.483 (0.116 - 2.011)	0.317
Sigmoid	1.374 (0.433 - 4.362)	0.590	1.194 (0.313 - 4.553)	0.795
<b>Differentiation</b>				
Poorly	1 ref.		1 ref.	
Moderately	0.524 (0.171 - 1.606)	0.258	4.105 (0.896 - 18.811)	0.069
Well	0.437 (0.146 - 1.313)	0.140	1.245 (0.357 - 4.334)	0.731
Unknown	0.586 (0.181 - 1.902)	0.374	1.163 (0.332 - 4.075)	0.814
<b>TNM stage</b>				
I	1 ref.		1 ref.	
II	1.765 (0.392 - 7.947)	0.459	0.779 (0.147 - 4.121)	0.769
III	1.778 (0.376 - 8.407)	0.468	0.665 (0.111 - 3.988)	0.656
IV	3.257 (0.671 - 15.802)	0.143	1.574 (0.256 - 9.675)	0.625
<b>Family history</b>				
No	1 ref.		1 ref.	
Yes	1.448 (0.675 - 3.110)	0.342	1.313 (0.478 - 3.607)	0.598
<b><math>\beta</math>-catenin</b>				
No	1 ref.		1 ref.	
Yes	2.586 (1.194 - 5.597)	0.016	3.842 (1.422 - 10.376)	0.008
<b>MSI status</b>				
MSS	1 ref.		1 ref.	
MSL	0.697 (0.283 - 1.718)	0.433	0.864 (0.284 - 2.624)	0.796
MSH	0.792 (0.286 - 2.193)	0.654	0.902 (0.245 - 3.328)	0.877
<b>Age of diagnose</b>				
< 50	1 ref.		1 ref.	
> 50	1.671 (0.802 - 3.480)	0.170	1.790 (0.703 - 4.559)	0.223

<sup>a</sup> 95% confidence interval.

**Figure 1.** Overall Survival Curves Relative to  $\beta$ -Catenin Status for all Colorectal Cancer Patients (N = 165)



Tumors with nuclear expression of  $\beta$ -catenin had a poorer prognosis compared to tumors with negative expression of nuclear  $\beta$ -catenin.

## 5. Discussion

Since detection and treatment in early stages of CRC can significantly improve patient survival, there has been great interest in developing diagnostic and prognostic biomarkers in the last few years. Thus, several studies have focused on the immunohistochemical pattern of  $\beta$ -catenin expression to elucidate its involvement in CRC. According to our results, patients with nuclear  $\beta$ -catenin positive expression were younger than patients with negative nuclear  $\beta$ -catenin expression (mean age of 49.25 years and 56.24 years, respectively) and this difference was statistically significant ( $P = 0.010$ ). In our study univariate and multivariate analysis showed that tumors with nuclear  $\beta$ -catenin expression had a poor prognosis compared with tumors without nuclear  $\beta$ -catenin expression. The study by Han et al. in 2006 validated tissue microarrays analysis for expression of  $\beta$ -catenin on tissue samples from normal colon mucosa, primary CRC and metastatic liver lesions. This study showed that decreased expression of  $\beta$ -catenin in a metastatic liver lesion may be a poor prognostic marker for CRC patients with liver metastasis (14).

While  $\beta$ -catenin signaling pathway plays a significant role in the carcinogenesis of colorectal cancer, the contribution of nuclear  $\beta$ -catenin in tumor development is far from clear. It is clear that dysfunction in the *Wnt*-signaling pathway and thereby nuclear accumulation of  $\beta$ -catenin is an important step in colorectal tumorigenesis (15, 16), however the details of this association and its contribution to the overall clinical outcome remain to be clarified (16). Wong et al. clearly showed that the expression of nuclear  $\beta$ -catenin increased notably during the development of normal mucous to carcinoma form (15). There is also evidence that CIN CRCs are frequently associated with high nuclear  $\beta$ -catenin expression (13).

A large-scale study by Wong et al. in 2004 documented that nuclear staining for  $\beta$ -catenin may serve as an additional factor to help discriminate colorectal adenocarcinomas from other tissue samples (17).

Previous studies have shown ambiguous results with respect to the distribution of nuclear, cytoplasmic and membranous staining of beta catenin with clinical outcome (18). Our results clearly showed a significant association between expression of nuclear  $\beta$ -catenin and overall survival in CRC patients. This finding is consistent with several studies that showed  $\beta$ -catenin overexpression in the nucleus predicts unfavorable survival in colorectal cancer (13-15, 17, 19-21). We also found that the nuclear  $\beta$ -catenin positive group with rectum tumors had a younger age of diagnosis ( $P = 0.004$ ) than the nuclear  $\beta$ -catenin negative group. We observed that the tumors in patients with positive  $\beta$ -catenin expression were predominantly located in the rectum. Recent papers on  $\beta$ -catenin indicating its prognostic value have been conflicting; with some studies reporting a good or no prognostic value (10, 11, 16, 22, 23), while others observed poorer clinical outcome in this regard (19, 21). Many issues may explain these contradictory results including intrinsic tumor heterogeneity, different immunohistochemical staining, visualization methods with varying degrees of sensitivity, and lack of standardization of what constitutes a "positive" or "negative" result. In many cases, nuclear  $\beta$ -catenin expression is mostly seen in the margin and not in the center of the tumor (10).

Although we found no significant association between patients with nuclear  $\beta$ -catenin positive and negative tumors based on TNM stage, Lugli and colleagues, in one study based on a tissue microarray analysis showed that increased expression of nuclear  $\beta$ -catenin and loss of membranous E-cadherin were related to tumor and lymph node invasion, the presence of vascular invasion and worse survival (24). Interestingly, Norwood et al. in a recent valuable paper showed that low levels of cytoplasmic  $\beta$ -catenin expression are associated with reduced survival in CRC patients as well as worse TNM staging in esophageal adenocarcinoma (10).

Our study showed that the nuclear  $\beta$ -catenin positive group had tumors located predominantly in the rectum site versus other sites and correlated with MSI status. In line with this result, Wangefjord et al. showed that high level of  $\beta$ -catenin expression was more frequent in rectum and sigmoid tumors and was also associated with MSI screening status (11). In line with our study, Tóth et al. reported that patients with dukes B2 stage colorectal tumors and loss of  $\beta$ -catenin expression were prone to metastatic spread. They also suggested that in Dukes B2 stage rectal cancers the loss of membranous expression of  $\beta$ -catenin is associated with a distant metastasis development (25). Another paper by Gomez-Millan et al. in 2013 revealed that nuclear  $\beta$ -catenin loss of expression was higher in the invasive margin in comparison with the tumor center. They also found that the mRNA levels of  $\beta$ -catenin are not associ-

ated with prognosis in this study (26). Wanitsuwan et al. revealed that the overall expression of  $\beta$ -catenin showed an association with better differentiation and early stage in CRC. However, they did not find a predictive value for nuclear  $\beta$ -catenin in CRC patients (23). Elzagheid and colleagues postulated that nuclear  $\beta$ -catenin expression provides extra information in predicting patient outcome in advanced CRC stages (27). Jaime Gomez-Millan et al. in 2014 evaluated whether preoperative Chemoradiotherapy (CRT) induces changes in the expression of  $\beta$ -catenin and if these related changes are associated with survival in locally advanced rectal cancer (LARC). In their study they observed that preoperative CRT in related subjects considerably alters the expression of nuclear  $\beta$ -catenin with 49% showing an increased expression after CRT treatment, 17% decreased expression and 34% no change;  $P = 0.001$ . They found that patients with overexpressed nuclear  $\beta$ -catenin after CRT treatment showed poor survival in comparison with patients with decreased nuclear  $\beta$ -catenin expression (28). The main limitation of this study was that the expression of  $\beta$ -catenin was assessed by immunohistochemistry (IHC). However, IHC is considered as a low sensitivity method in comparison with other techniques including real-time polymerase chain reaction (PCR) and fluorescence in situ hybridization (FISH).

In summary, our findings indicate that nuclear  $\beta$ -catenin expression was associated with MSI status and tumor location in Iranian CRC patients. We also observed an association between nuclear  $\beta$ -catenin expression and poor overall survival. In conclusion,  $\beta$ -catenin nuclear expression is strongly correlated with colorectal carcinogenesis, and can potentially serve as a new prognostic indicator independent of stage and grade. The clinical use of  $\beta$ -catenin as a prognostic factor needs to be investigated in larger patient cohorts.

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## Authors' Contributions

Study concept and design: Mahsa Molaei, Ehsan Nazemalhosseini-Mojarad. Lab working, interpreted the clinical data and data collection: Hanieh Mirtalebi, Shohreh Almasi, Vahid Chaleshi, Roya Kishani Farahani, Peyman Tarban. Analysis and interpretation of data: Ehsan Nazemalhosseini-Mojarad, Seyed Mohammad Hossein Kashfi. Drafting of the manuscript: Ehsan Nazemalhosseini-Mojarad, Seyed Mohammad Hossein Kashfi. Critical revision of the manuscript for important intellectual content: Peter J.K. Kuppen, Mohammad Reza Zali. Statistical analysis: Ehsan Nazemalhosseini-Mojarad. Administrative, technical, and material support: Mohammad Reza Zali, Study supervision: Ehsan Nazemalhosseini-Mojarad.

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