JBUON 2017; 22(2): 431-436
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
E-mail: editorial_office@jbuon.com

ORIGINAL ARTICLE

Assessing the clinical utility of Wnt pathway markers in colorectal cancer

Georgios Veloudis[1], Apostolos Pappas[2], Stavros Gourgiotis[1], Evangelos Falidas[1], Nikoletta Dimitriou[3], Ioannis Karavokiros[3], Anastasios Aggelou[3], Vasileios Komborozos[4], Constantina Petraki[5], Panagiotis Menounos[6], Ioannis Bramis[3], Elias Bastounis[3], Emmanouel Pikoulis[3]

[1]Gastroenterology Department, "Sismanogleio" General Hospital of Athens, Athens; [2]First Surgical Department, "417 NIMTS" Military Veterans' Fund Hospital of Athens, Athens; [3]First Department of Surgery, "Laikon" General Hospital, Athens Medical School, National and Kapodistrian University of Athens, Athens; [4]Third Surgical Department, "Evangelismos" Hospital of Athens, Athens; [5]Department of Pathology, "Evangelismos" Hospital of Athens, Athens; [6]Molecular Biology Laboratory, 401 General Army Hospital of Athens, Athens, Greece

Summary

Purpose: *β-catenin and AXIN2 play an important role in the Wnt signaling pathway. The aim of this study was to investigate β-catenin and AXIN2 expression in colorectal cancer (CRC) and relate these findings with patients' clinicopathological features and prognosis.*

Methods: *57 consecutive patients with surgically treated CRC were included in this study. Quantitative PCR and immunohistochemistry (IHC) analyses were performed to characterize the expression of the aforementioned markers in CRC tissues.*

Results: *β-catenin overexpression in the nucleus was associated with advanced N stage CRCs (p=0.04). Multivariate Cox regression analysis showed that β-catenin overexpression is an independent prognostic factor for overall survival (OS). A positive correlation between β-catenin location and AXIN2 mRNA was observed.*

Conclusions: *Nuclear β-catenin is a valuable prognostic factor. AXIN2 is a component of the "Destruction Complex" and also a Wnt target gene. However, the clinical importance of AXIN2 expression in CRC remains unclear.*

Key words: *AXIN2, β-catenin, colorectal cancer, prognosis*

Introduction

CRC is the second most common cancer worldwide with more than one million new cases per year [1]. The cancer-related mortality is about 694,000 deaths per year worldwide [1] with about 75% of them being sporadic cases caused by somatic mutations [2]. The majority of the sporadic cases show mutations in genes involved in Wnt/β-catenin signaling, such as the tumor suppressor adenomatous polyposis coli (APC) or β-catenin. Mutations in these genes lead to an aberrant activation of the Wnt/β-catenin pathway [3,4].

The Wnt pathway is one of the fundamental mechanisms that regulate tissue homeostasis. [5,6]. In the presence of Wnt ligands, a cascade of events stimulates several intra-cellular signaling pathways, including the canonical and the non-canonical Wnt pathway which can be further divided into the Planar Cell Polarity and the Wnt/Ca2+ pathway [7]. β-catenin regulation is the hallmark of the canonical Wnt pathway. Without Wnt signaling, cytosolic β-catenin is rapidly phosphorylated by a complex of proteins, comprised of AXIN, APC, Glycogen Synthase Kinase 3 (GSK3), and Casein Kinase 1 (CK1) [8,9].

A plethora of studies have shown that mu-

Correspondence to: Emmanouel Pikoulis, MD, PhD. 17th Agiou Thoma str, 11527, Athens, Greece. Tel:+30 6945463193,
E-mail: mpikoul@med.uoa.gr
Received: 11/08/2016; Accepted: 04/10/2016

tations in the Wnt components and subsequent activation of the pathway play an important role in the process of colon tumor genesis. Inhibition of β-catenin degradation leads to accumulation in the cytosol and translocation to the nucleus where it interacts with members of the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors and activates target genes which influence cell differentiation, proliferation and migration [10-12]. Nonetheless reports about the prognostic significance of β-catenin in CRC have been contradictory. Researchers argue whether nucleus or cytoplasmic β-catenin is a better marker of Wnt activity [13,14].

AXIN is a key component of the destruction complex and a major control point for β-catenin stability [15]. There are two AXIN proteins: AXIN1 and AXIN2. AXIN2 has high structural similarities and is considered functionally equivalent to AXIN1. However, AXIN1 is more widely expressed, whereas AXIN2 shows a more restricted expression pattern and serves also as a transcriptional target of β-catenin-dependent Wnt signaling [16,17]. Although AXIN2 appears to serve as a negative regulator of canonical Wnt signaling, there are studies that support the hypothesis that AXIN2 may in fact function as an oncogene rather than a tumor suppressor [18].

The purpose of the present study was to examine the immunohistochemical expression of cytoplasmic and nuclear β-catenin and the expression of AXIN2 at the messenger ribonucleic acid (mRNA) and protein level and correlate these findings with clinicopathological parameters and patients survival.

Methods

A total of 57 consecutive patients with CRC, surgically treated at our department were included in this study. None of these patients had received chemotherapy or irradiation pre-operatively. In order to avoid RNA degradation and preserve RNA stability, fresh frozen sections were stored in RNA later tissue preservative (Ambion, USA). Histopathological examination was performed in formalin-fixed paraffin embedded biopsy sections that were cut at 5μm thickness, followed by standard hematoxylin and eosin staining. The Hospitals' review board approved this study and written informed consent was obtained when available from each subject or from his/her guardian.

Immunohistochemistry (IHC)

β-catenin and AXIN2 protein expression was evaluated by IHC analysis in formalin- fixed paraffin-embedded tissue sections. The antibodies used for β-catenin and AXIN2 proteins were the following: a) β-catenin: antibody H-102, class: rabbit polyclonal; epitope: 680- 781 (Santa Cruz, CA, USA) and b) AXIN2: antibody H-260, class: rabbit polyclonal; epitope 541-800 (Santa Cruz, CA, USA). Five μm paraffin tissue sections were mounted on poly-L-lysine-coated slides, dewaxed, dehydrated and incubated with the primary antibody at 1/100 dilution at 4°C for 2 hrs at a concentration of 0.04 μg/ml. Biotin-conjugated secondary antibody was added at a 1/200 dilution for 1 hr at room temperature. For color development, 3'-diaminobenzidine tetrachloride (DAB) and hematoxylin were used as a counterstain according to the manufacturer's instructions. Incubating paraffin sections in the absence of the primary antibody tested the specificity of each of the primary antibodies used in this study. The staining was scored according to the proportion and intensity. The proportion score represents the estimated fraction of positive cells (< 30%, 30-50% and >50%), while the intensity score represents their average staining intensity (negative, weak, intermediate, and strong). The final score for each tumor was calculated by adding these two scores. The median value was used to categorize samples into high and low expression levels. In case of β-catenin IHC detection was examined separately for the cytoplasmic and nuclear localization of the protein.

RNA extraction

Total RNA was isolated from all samples using the RNeasy Midi Kit (Qiagen, Germany), according to the manufacturer's instructions. RNA concentration and quality were assessed by spectrophotometry and by the use of the Agilent 2100 Bioanalyzer with Agilent 2100 expert software system according to the manufacturer's instructions (Agilent, USA). RNasey free polypropylene thin-wall microfuge tubes and pipette tips were used for all RNA preparations and assays (Perkin Elmer Co, Roche, Switzerland). Quantitative PCR (qPCR) was performed with a DNA Engine Opticon device according to the manufacturer's instructions (MJ Research). For each tumor sample, measurements were repeated three times and expression data were reported as column bars representing the relative fold change in mRNA levels for each of the designated genes as related to the paired adjacent normal tissue sample. The mRNA expression of tumors was classified as being higher or lower than the median value.

Statistics

Statistical analyses were performed using the x^2-test and the Kruskal Wallis test for K-independent samples where appropriate. Fisher's exact test was used as an additional method for non-parametric test procedures in small data sets. Disease-free (DFS) and OS were estimated by the Kaplan-Meier method and compared with the log rank test. Multivariate Cox regression

analysis was used to select independent prognostic factors. Statistical analyses were performed with SPSS 8.0 software (SPSS Inc, Chicago, Ill, USA). Two-sided tests were used to calculate probability values (p). Statistical significance was set at p<0.05.

Results

Study analysis included 41 colon and 16 rectal adenocarcinoma patients. Seven (12.3%) patients were in stage I, 19 (33.3%) in stage IIA, 3 (5.3%) in stage IIIA, 13 (22.8%) in stage IIIB, 5 (8.8%) in stage IIIC, and 10 (17.5%) in stage IV. The majority of patients (66.7%) had grade II tumors.

Cytoplasmic and focal nuclear accumulation of β-catenin was observed in 88% (50/57) and 25% (14/57) of patients respectively. Cytoplasmic accumulation of AXIN2 was observed in 33% (19/57) of the patients and high AXIN2 mRNA in 51% (29/57) of the patients.

No correlation was found between cytoplasmic β-catenin and AXIN2 protein/mRNA and tumor location, grade and TNM stage. Tumors with reduced or absent nuclear β-catenin expression were less likely to develop regional lymph node metastases (Table 1). Furthermore, there was no association between nuclear β-catenin expression and tumor location, grade and TNM stage (Table 1).

The correlation between cytoplasmic and nuclear β-catenin expression was also evaluated and showed that accumulation of β-catenin in the cytoplasm was particularly enhanced in those cases with low β-catenin expression in the nucleus (Table 2).

AXIN2 mRNA showed a significant association with β-catenin expression. AXIN2 mRNA level was higher in low cytoplasmic and high nuclear β-catenin tumors (Table 3).

Univariate analyses showed that high nuclear β-catenin expression was significantly associated with a worse OS (Figure 1). No correlation was found with DFS.

When standard clinicopathological variables were adjusted for by Cox modeling, high nuclear β-catenin expression was still an independent predictor of short OS (Table 4).

No significant association was found between

Table 1. Comparison of pathological parameters with nuclear β-catenin expression

Parameters	*Nuclear β-catenin (Low) n (%)*	*Nuclear β-catenin (High) n (%)*	*p value*
Tumor location			0.73
Right colon	13 (30.2)	6 (42.9)	
Left colon	17 (39.5)	5 (35.7)	
Rectum	13 (30.2)	3 (21.4)	
T			0.84
T1	4 (9.3)	2 (14.3)	
T2	4 (9.3)	1 (7.1)	
T3	35 (81.4)	11 (78.6)	
N			0.04
N0	19 (44.2)	8 (57.1)	
N1	20 (46.5)	2 (14.3)	
N2	4 (9.3)	4 (28.6)	
M			0.20
M0	38 (88.4)	10 (71.4)	
M1	5 (11.6)	4 (28.6)	
TNM stage			0.45
I	5 (11.6)	2 (14.3)	
IIA	13 (30.2)	6 (42.9)	
IIIA	3 (7)	0 (0)	
IIIB	12 (27.9)	1 (7.1)	
IIIC	4 (9.3)	1 (7.1)	
IV	6 (14)	4 (28.6)	
Grade of differentiation			0.51
Well	1 (2.3)	1 (7.1)	
Moderate	28 (65.1)	10 (71.4)	
Poor	14 (32.6)	3 (21.4)	

Table 2. Correlation between nuclear and cytoplasmic β-catenin

	β-catenin nuclear		*p value*
	n (%)	*n (%)*	
β-catenin cytoplasmic	*Low*	*High*	0.05
Low	3 (7)	4 (28.6)	
High	40 (93)	10 (71.4)	

Table 3. Correlation between β-catenin and AXIN-2 mRNA level

	AXIN-2 mRNA level	*p value*
β-catenin cytoplasmic		0.03
Low	12.2 [5.7-16.6]	
High	3.1 [1.8-8.3]	
β-catenin nuclear		0.001
Low	2.9 [1.7-4.5]	
High	10.8 [8.3-16.3]	

Table 4. Multivariate Cox regression analysis of nuclear β-catenin

Variables	*p value*	*HR*	*95% CI*
Age	0.31	1.03	0.97-1.09
Gender	0.44	1.54	0.52-4.60
Location	0.23	2.21	0.60-8.13
Grade	0.05	0.28	0.08-1.04
TNM stage	<0.01	0.03	0.00-0.38
Nuclear β-catenin	0.02	3.86	1.24-11.99

HR: hazard ratio, CI: confidence interval

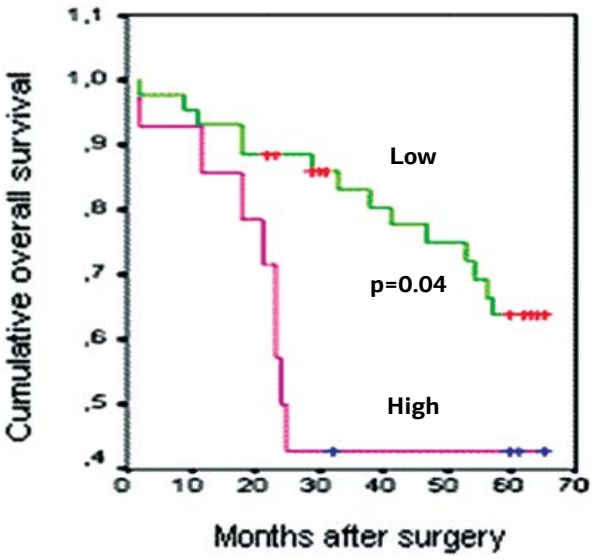

Figure 1. Kaplan-Meier overall survival with regard to nuclear β-catenin expression.

AXIN2 and cytoplasmic β-catenin expression and patient OS and DFS.

Discussion

The Wnt/β-catenin signaling cascade is an important pathway involved in initiation and maintenance of CRC [19]. Regulation of this pathway is realized through the level of β-catenin protein in the nucleus. β-catenin maintains a low cytoplasmic concentration through the destruction complex when the Wnt signaling pathway is inactivated. Otherwise, the destruction complex is dissolved and β-catenin accumulates in the cell and undergoes translocation to the nucleus [13].

To date, correlations between immunohistochemically detected expression of β-catenin in CRC and prognosis remain highly variable. In the present study we have shown that nuclear overexpression of β-catenin was associated with impaired survival in CRC patients (p=0.04). The results of our small series of patients were in concordance with a recent meta-analysis of 18 studies, including 3665 cases, which showed that β-catenin overexpression in the nucleus rather than in the cytoplasm, influenced survival of CRC patients [13]. On the contrary, survival analysis by Bruun et al, of 903 CRC patients, showed that membranous or cytosolic but not nuclear β-catenin independently predicted poor outcome. Also only membranous and cytosolic β-catenin was correlated with the histopathologic grade of the tumor [14]. In a meta-analysis by Chen et al., researchers concluded that β-catenin overexpression in the nucleus was not associated with the differentiation grade, lymph node status or depth of invasion. No significant association was shown between β-catenin overexpression in the cytoplasm and Dukes' stages and lymph node status [9]. On the contrary, in our study nuclear β-catenin overexpression was associated with lymph node metastases (p=0.04). Similar results were reported by Luigli et al., which associated overexpression of nuclear β-catenin with higher N stage [20].

These discrepancies might be attributed to methodological differences and/or reflect the complexity surrounding the regulation of β-catenin translocation from the cytoplasm to the nucleus. The movement of β-catenin between the two intra-cellular compartments is a dynamic process orchestrated by several molecules such as BCL-9, APC and AXIN [21-23]. Studies have also shown that nuclear accumulation of β-catenin requires activation of Rac1 and subsequent β-catenin phosphorylation [24].

Although the accumulation of β-catenin in the cytoplasm and nuclear translocation appear to be highly correlated processes, immunohistochemical data from CRCs series have provided divergent results. While some researchers have failed to show a correlation between expression of nuclear and cytoplasmic β-catenin, others have described the presence of a positive correlation [25,26]. In our study, cytoplasmic and nuclear deposits of β-catenin appeared to be mutually exclusive, a finding that may support the notion of a common β-catenin pool shared between the two major cell compartments.

Furthermore, our study showed a correlation between β-catenin location and AXIN2 mRNA. Our results contrast previous studies reporting that β-catenin localization was not affected by the expression of AXIN2 [2]. AXIN2 is a key regulator in Wnt/β-catenin signaling and a target gene at the same time. It acts in a negative-feedback loop to regulate the stability of β-catenin [27,28]. The AXIN proteins form a multiprotein complex with APC and GSK-3β and conduct the degradation of β-catenin. Thus, by serving as a negative feedback mechanism for the regulation of Wnt pathway, AXIN could act as tumor suppressor, inhibiting cell dedifferentiation and proliferation [29,30].

On the contrary a study by Wu et al. involving CRC cell lines showed that AXIN 2 acts as a potent promoter of cancer behavior by upregulating the activity of the transcriptional repressor Snail 1, thereby promoting epithelial to mesenchymal transition (EMT) and driving metastatic activity [31].

The role of AXIN2 in CRC progression and

survival remains elusive and unclear. In agreement with our results, a study by Schaal et al. in a cohort of 280 CRC patients, failed to show any association between AXIN2 and tumors' stage, histopathological grade and patients' survival [2]. These discrepancies could reflect the in vivo complex correlations between AXIN2 and the components of the Wnt and EMT pathway.

Conclusion

This study showed that β-catenin is key factor in the Wnt pathway and its nuclear detection appears to serve as valuable prognostic factor of CRC patients. The inverse relationship between cellular and nuclear β-catenin supports the notion of the presence of a common β-catenin pool regulated by the Wnt components. Besides, being a component of the "Destruction Complex", AXIN2 is also a Wnt target gene. However, the clinical importance of evaluating AXIN2 expression in CRC remains unclear.

Conflict of interests

The authors declare no confict of interests.

References

1. Ferlay J, Soerjomataram I, Dikshit R et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 2015;136:E359-386.
2. Schaal U, Grenz S, Merkel S et al. Expression and localization of axin 2 in colorectal carcinoma and its clinical implication. Int J Colorectal Dis 2013;28:1469-1478.
3. Powell SM, Zilz N, Beazer-Barclay Y et al. APC mutations occur early during colorectal tumorigenesis. Nature 1992;359:235-237.
4. Schneikert J, Behrens J. The canonical Wnt signalling pathway and its APC partner in colon cancer development. Gut 2007;56:417-425.
5. Logan CY, Nusse R. The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol 2004;20:781-810.
6. Rao TP, Kuhl M. An updated overview on Wnt signaling pathways: a prelude for more. Circ Res 2010;106:1798-1806.
7. Komiya Y, Habas R. Wnt signal transduction pathways. Organogenesis 2008;4:68-75.
8. Gordon MD, Nusse R. Wnt signaling: multiple pathways, multiple receptors, and multiple transcription factors. J Biol Chem 2006;281:22429-22433.
9. Kimelman D, Xu W. beta-catenin destruction complex: insights and questions from a structural perspective. Oncogene 2006;25:7482-7491.
10. Segditsas S, Tomlinson I. Colorectal cancer and genetic alterations in the Wnt pathway. Oncogene 2006;25:7531-7537.
11. Wu WK, Wang XJ, Cheng AS et al. Dysregulation and crosstalk of cellular signaling pathways in colon carcinogenesis. Crit Rev Oncol Hematol 2013;86:251-277.
12. White BD, Chien AJ, Dawson DW. Dysregulation of Wnt/beta-catenin signaling in gastrointestinal cancers. Gastroenterology 2012;142:219-232.
13. Chen Z, He X, Jia M et al. Beta-catenin overexpression in the nucleus predicts progress disease and unfavourable survival in colorectal cancer: a meta-analysis. PLoS One 2013;8:e63854.
14. Bruun J, Kolberg M, Nesland JM et al. Prognostic Significance of beta-Catenin, E-Cadherin, and SOX9 in Colorectal Cancer: Results from a Large Population-Representative Series. Front Oncol 2014;4:118.
15. Fagotto F, Jho E, Zeng L et al. Domains of axin involved in protein-protein interactions, Wnt pathway inhibition, and intracellular localization. J Cell Biol 1999;145:741-756.
16. Leung JY, Kolligs FT, Wu R et al. Activation of AXIN2 expression by beta-catenin-T cell factor. A feedback repressor pathway regulating Wnt signaling. J Biol Chem 2002;277:21657-21665.
17. Chia IV, Costantini F. Mouse axin and axin2/conductin proteins are functionally equivalent in vivo. Mol Cell Biol 2005;25:4371-4376.
18. Hadjihannas MV, Bruckner M, Behrens J. Conductin/axin2 and Wnt signalling regulates centrosome cohesion. EMBO Rep 2010;11:317-324.
19. Scholer-Dahirel A, Schlabach MR, Loo A et al. Maintenance of adenomatous polyposis coli (APC)-mutant colorectal cancer is dependent on Wnt/beta-catenin signaling. Proc Natl Acad Sci U S A 2011;108:17135-17140.
20. Lugli A, Zlobec I, Minoo P et al. Prognostic significance of the wnt signalling pathway molecules APC, beta-catenin and E-cadherin in colorectal cancer: a tissue microarray-based analysis. Histopathology 2007;50:453-464.
21. Townsley FM, Cliffe A, Bienz M. Pygopus and Legless target Armadillo/beta-catenin to the nucleus to enable its transcriptional co-activator function. Nat Cell Biol 2004;6:626-633.
22. Henderson BR. Nuclear-cytoplasmic shuttling of APC regulates beta-catenin subcellular localization and turnover. Nat Cell Biol 2000;2:653-660.
23. Cong F, Varmus H. Nuclear-cytoplasmic shuttling of Axin regulates subcellular localization of beta-catenin. Proc Natl Acad Sci U S A 2004;101:2882-2887.
24. Wu X, Tu X, Joeng KS et al. Rac1 activation controls nuclear localization of beta-catenin during canonical Wnt signaling. Cell 2008;133:340-353.

25. Filiz AI, Senol Z, Sucullu I et al. The survival effect of E-cadherin and catenins in colorectal carcinomas. Colorectal Dis 2010;12:1223-1230.
26. Bondi J, Bukholm G, Nesland JM, Bukholm IR. Expression of non-membranous beta-catenin and gamma-catenin, c-Myc and cyclin D1 in relation to patient outcome in human colon adenocarcinomas. APMIS 2004;112:49-56.
27. Jho EH, Zhang T, Domon C et al. Wnt/beta-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway. Mol Cell Biol 2002;22:1172-1183.
28. Lustig B, Jerchow B, Sachs M et al. Negative feedback loop of Wnt signaling through upregulation of conductin/axin2 in colorectal and liver tumors. Mol Cell Biol 2002;22:1184-1193.
29. Lee E, Salic A, Kruger R, Heinrich R, Kirschner MW. The roles of APC and Axin derived from experimental and theoretical analysis of the Wnt pathway. PLoS Biol 2003;1:E10.
30. Mazzoni SM, Fearon ER. AXIN1 and AXIN2 variants in gastrointestinal cancers. Cancer Lett 2014;355:1-8.
31. Wu ZQ, Brabletz T, Fearon E et al. Canonical Wnt suppressor, Axin2, promotes colon carcinoma oncogenic activity. Proc Natl Acad Sci U S A 2012;109:11312-11317.