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Title:

Canonical Wnt/β-catenin signaling pathway is dysregulated in patients with primary and secondary myelofibrosis

Auhors:

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MicroAbstract:

Activation of Wnt/β-catenin signaling pathway is associated with malignant transformation, development of fibrosis and angiogenesis. We analyzed β-catenin mRNA expression in bone marrows of 29 patients with primary (PMF), four with secondary myelofibrosis (SMF) and 16 controls using qRT-PCR. B-catenin expression is increased in both PMF and SMF and might potentiate anemia.

Abstract:

Introduction: B-catenin is a central effector molecule of canonical Wnt signaling pathway. It is important for maintenance of stem cell homeostasis and its aberrant activation has been implicated in a wide array of malignant hematological disorders. There are few reports suggesting its dysregulation in Philadephia chromosome negative myeloproliferative neoplasms (Ph- MPNs).

Patients and methods: We analyzed β-catenin mRNA expression in bone marrow (BM) aspirates of 29 patients with primary (PMF) and four patients with secondary, post Ph- MPN, myelofibrosis (SMF) using qRT-PCR. Control group consisted of 16 BM aspirates from patients with limited-stage aggressive Non-Hodgkin lymphoma without BM involvement. We compared relative gene expression with clinical and hematological parameters.

Results: B-catenin relative expression differed significantly among groups (p=0.0002), it was significantly higher in patients with both PMF and SMF than in control group, but did not differ between PMF and SMF patients. Negative correlation was found regarding hemoglobin level in PMF (p=0,017). No association according to JAK2 V617F mutational status or JAK2 V617F allele burden was detected.

Conclusion: Present study shows for the first time that β-catenin mRNA expression is increased in patients with both PMF and SMF and its upregulation might potentiate anemia. Number of inflammatory cytokines associated with PMF are capable of mediating their effects through increased β-catenin expression. Accordingly, β-catenin can induce expression of number of genes implicated in processes of cell cycle control, fibrosis and angiogenesis which are central to the PMF pathogenesis. Therefore, β-catenin may represent interesting new therapeutic target in these diseases.

Keywords:

Wnt; β-catenin; primary myelofibrosis; secondary myelofibrosis; JAK2 V617F

Introduction:

Primary myelofibrosis (PMF) is a Philadelphia chromosome negative myeloproliferative neoplasm (Ph- MPN)[1] driven by clonal expansion of pluripotent hematopoietic stem cell.[2] Proliferating clone and bone marrow stroma[3] produce inflammatory cytokines leading to reactive bone marrow fibrosis, increased angiogenesis and subsequent development of extramedullary hematopoiesis. Although no disease-causing mutation has been recognized, JAK2, MPL or calreticulin mutations are present in majority of patients.[4] Disease can manifest differently in individual patients with varying number of myeloid lineages proliferation or cytopenias, marked hepato and splenomegaly with associated complications and prominent constitutional symptoms.

PMF related Ph- MPNs like polycythemia rubra vera (PRV) and essential thrombocytosis (ET) share common biological and clinical features with PMF and can evolve to secondary myelofibrosis (SMF) during course of disease. PMF and post PRV / post ET SMF have different molecular backgrounds; PMF harbors larger number of mutated genes while SMF more closely mirrors the disease of origin.[5, 6]

Homeostasis of both healthy and diseased human tissues is regulated by a stem cell network of signaling cascades where canonical Wnt signaling cascade plays a pivotal role.[7] Binding of canonical Wnt ligands (Wnt1, Wnt3a, Wnt8) with receptor Frizzled induces membranous complex formation with low-density-lipoprotein receptor related protein 5/6 (LRP5/6) and recruits intracellular proteins Dishevelled and Axin to plasma membrane.[8] Without canonical Wnt stimulation, Axin resides in a complex with adenomatous polyposis coli (APC), glycogen synthase kinase 3 (GSK3) and casein kinase 1 (CK1). Complex captures central effector molecule of canonical Wnt signaling – β-catenin which leads to its phosphorylation, ubiquitination and subsequent degradation. Upon canonical Wnt stimulation, β-catenin is released from aforementioned protein complex, pairs with the lymphoid enhancer factor/T cell factor (LEF/TCF), enters nucleus and induces transcriptional activation of target genes involved in stem cell maintenance, expansion and lineage specification in both embryonic and adult tissues.[9] Aberrant activation of canonical Wnt/β-catenin signaling increases propensity for cell malignant transformation and has been implicated in a wide array of malignant hematological disorders like multiple myeloma[10], chronic lymphocytic leukemia[11], chronic myelogenous leukemia (CML)[12], acute myeloid leukaemia[13] etc. There are few reports suggesting its dysregulation in Ph-MPNs[14, 15] with PMF patients receiving only limited attention.

Aim of this study was to analyze the expression of canonical Wnt effector β-catenin in bone marrow aspirates of patients with PMF and SMF through mRNA expression. We compared relative gene expression with clinical and hematological parameters.

Patients and methods:

Bone marrow aspirates of 29 patients with PMF and four patients with SMF who were diagnosed in our institution from 2004 to 2015 were analyzed. The diagnosis was established according to the current WHO / IWG-MRT criteria[16, 17] and the degree of bone marrow fibrosis was graded according to the current European consensus.[18] Control group consisted of 16 age and sex matched bone marrow aspirates obtained from patients with limited-stage aggressive Non-Hodgkin lymphoma without bone marrow involvement. All patients and controls provided written informed consent for bone marrow cryopreservation and molecular studies. The study was approved by the Institutional Review Board.

Mononuclear cells were isolated from bone marrow aspirates using Histopaque (Sigma, St. Louis, MO, USA; density 1.077 g/mL) and preserved in liquid nitrogen using DMSO until needed. Total RNA was extracted using Trizol reagent (TriPure; Roche Mannheim, Germany), reversely transcribed

to cDNA (MuLV Reverse Transcriptase; Applied Biosystems, Foster City, CA) and amplified by quantitative PCR using CTNNB1 TaqMan gene expression assay (Hs00355049_m1 Thermo Fisher Scientific) in an ABI Prism 7300 Sequence Detection system (Applied Biosystems). QRT-PCR was performed in duplicate for each sample. B-catenin relative expression was calculated as ΔCT values using *abl* as the reference gene ($CT_{\beta\text{-}catenin}$ - CT_{abl}). JAK2 V617F mutation analysis and transcript levels quantification were performed according to previously published methods.[19, 20]

The normality of data distrubution was tested using the Shapiro-Wilk test. ΔCT values were compared using the Kruskal-Wallis one-way analysis of variance and corresponding post-hoc tests. Association between parameters was tested using the Spearman rank correlation or Mann Whitney U test where appropriate. All statistical tests were two-sided and P values <0.05 were considered significant. Analyses were performed using MedCalc Statistical Software version 16.2.0 (MedCalc Software bvba, Ostend, Belgium).

Results:

There were total of 49 patients and controls analyzed, 29 with PMF, four with SMF (three post PRV SMF, one post ET SMF) and 16 in control group. There were 31 (63%) male and 18 (37%) female patients, median age was 69 years. There were no significant differences in age or sex in target and control groups. Clinical characteristics of patients with PMF and SMF are shown in Table 1.

B-catenin relative expression differed significantly between groups (p=0.0002). B-catenin was significantly higher expressed in both patients with PMF (median ΔCT -4.65) and SMF (median ΔCT -4.27) than in control group (median ΔCT -3.39), but the expression did not differ significantly between PMF and SMF patients as shown in Figure 1.

In patients with PMF, β-catenin relative expression correlated negatively with hemoglobin level (p=0,017), but no association according to age, sex, degree of bone marrow fibrosis, the spleen or the liver size, leukocyte or platelet counts, MCV, RDW, LDH, serum iron, TIBC, transferrin saturation, ferritin or CRP was detected. Also, no association between β-catenin relative expression and *JAK2* V617F mutational status was detected; JAK2 allele burden in mutated patients did not correlate with β-catenin expression (analyzed in 12 patients).

Discussion:

Present study shows for the first time that β-catenin mRNA expression is increased in patients with both primary and secondary (post Ph- MPN) myelofibrosis. We identified three previous studies investigating β-catenin expression in PMF with neither of them focusing primarily on this disease, SMF has not been investigated previously. First study was done by Serinsöz et al[13] who used qRT-PCR method to demonstrate increased β-catenin expression in PMF patients in comparison to CML but not in comparison to control group. It is to point out that mentioned study used RNA extracted from paraffin embedded bone marrow biopsies. Also, patients with reactive hyperplasia of megakaryopoiesis and/or erythropoiesis were used as control group. Second study was done by Jauregui et al[14] who used immunohistochemistry to demonstrate lower expression of β-catenin in megakaryocytes of PMF and CML patients in comparison to PRV and ET. Third study was done by Geduk et al[15] who used immunohistochemistry to analyze β-catenin expression in Ph- MPN subsets distinguishing expression in myeloid cells, megakaryocytes and vascular endothelial cells. They showed lower expression of β-catenin in megakaryocytes of PMF patients in comparison to ET and PRV (there was no difference between PMF and control group), no difference in β-catenin expression in myeloid cells among tested subsets and higher β-catenin expression in vascular endothelial cells of

PMF patients in comparison to PRV, ET and control group. Authors also reported positive correlation between β-catenin expression in megakaryocytes and hemoglobin level (for a whole Ph- MPN group represented with 43 ET, seven PRV and 16 PMF patients that differ in disease biology and hemoglobin levels) which is in contrast with our finding of negative correlation with hemoglobin level in pure PMF group. Our results suggest that upregulation of canonical Wnt/β-catenin signaling might potentiate anemia. This novel observation has clinical importance as lower hemoglobin levels bear adverse prognostic significance in PMF (but not other Ph- MPNs). In general, our findings are in support of previous observations that β-catenin may have important role in PMF.

It was previously shown by Liu et al[21] that JAK2 specific blockade can decrease β-catenin expression in human acute T cell leukemia Jurkat cells and in human erythroleukemia HEL cells. This effect can be attenuated by silencing beta-TrCP with specific shRNA, identifying beta-TrCP as cross-talk gene between JAK/STAT and Wnt/β-catenin signaling pathways. Expression of β-catenin in our study shows no association with either JAK2 V617F mutational status or JAK2 V617F allele burden which is in line with previous observations regarding Ph- MPNs[15, 22] suggesting that β-catenin expression could independently contribute to pathogenesis of Ph- MPNs but future studies are needed.

Canonical Wnt/β-catenin signaling is a part of larger stem cell signaling network where different cytokine signaling cascades cooperate or antagonize in a context dependent manner.[23] The foundation of this network comprises of Wnt, FGF, Notch, TGFβ/BMP, and Hedgehog signaling cascades.[24] Elements of the network are mutually regulated and play a central role in stem cell fate determination by governing processes of self-renewal, proliferation and differentiation which are central to fetal and adult tissues homeostasis and carcinogenesis. Number of inflammatory cytokines associated with PMF are capable of mediating their effects through increased β-catenin expression (reported in different contexts), including TGFβ1,[25, 26] FGF[27] and PDGF.[28, 29] Accordingly, β-catenin can induce expression of number of genes implicated in processes of cell cycle control (c-MYC, Cyclin D1),[30-32] fibrosis (COL1, TGFβ, ET-1, CCN2)[33, 34] and angiogenesis (IL-8, VEGF).[35, 36] Which of aforementioned interactions are relevant in pathogenesis of PMF remains to be elucidated with further studies. Since clonal proliferation associated with bone marrow fibrosis and increased angiogenesis are central features of PMF and SMF, β-catenin may represent interesting new therapeutic target in these diseases.

Conclusion:

Both PMF and SMF patients have increased β-catenin expression in their bone marrows. Activation of canonical Wnt/β-catenin signaling might potentiate anemia in PMF and may contribute to malignant and fibrogenic potential of these diseases. This could provide interesting new therapeutic targets.

Clinical Practice Points:

- Activation of Wnt/β-catenin signaling pathway promotes malignant transformation and development of fibrosis and angiogenesis.
- B-catenin contributes to pathogenesis of various hematological malignancies, including multiple myeloma and chronic myelogenous leukemia where its inhibition has been actively investigated.
- The present study demonstrated increased β-catenin mRNA expression in bone marrows of patients with both primary (PMF) and secondary myelofibrosis (SMF) developing from previous Philadelphia chromosome negative myeloproliferative neoplasm.
- Higher β-catenin expression is associated with lower hemoglobin level which bears negative prognostic significance in PMF.
- Our results are providing additional evidence for role of β-catenin in pathogenesis of PMF and SMF which may help in recognizing new therapeutic targets in these diseases.

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Literature:

1. Vardiman JW, Thiele J, Arber DA, et al. The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. *Blood.* 2009;114:937-951.

2. Buschle M, Janssen JW, Drexler H, Lyons J, Anger B, Bartram CR. Evidence for pluripotent stem cell origin of idiopathic myelofibrosis: clonal analysis of a case characterized by a N-ras gene mutation. *Leukemia.* 1988;2:658-660.

3. Martinaud C, Desterke C, Konopacki J, et al. Osteogenic Potential of Mesenchymal Stromal Cells Contributes to Primary Myelofibrosis. *Cancer research.* 2015;75:4753-4765.

4. Tefferi A, Lasho TL, Finke CM, et al. CALR vs JAK2 vs MPL-mutated or triple-negative myelofibrosis: clinical, cytogenetic and molecular comparisons. *Leukemia.* 2014.

5. Mills KI, McMullin MF. Mutational spectrum defines primary and secondary myelofibrosis. *Haematologica.* 2014;99:2-3.

6. Brecqueville M, Rey J, Devillier R, et al. Array comparative genomic hybridization and sequencing of 23 genes in 80 patients with myelofibrosis at chronic or acute phase. *Haematologica.* 2014;99:37-45.

7. Katoh M, Katoh M. WNT signaling pathway and stem cell signaling network. *Clinical cancer research : an official journal of the American Association for Cancer Research.* 2007;13:4042-4045.

8. Grumolato L, Liu G, Mong P, et al. Canonical and noncanonical Wnts use a common mechanism to activate completely unrelated coreceptors. *Genes & development.* 2010;24:2517-2530.

9. Grigoryan T, Wend P, Klaus A, Birchmeier W. Deciphering the function of canonical Wnt signals in development and disease: conditional loss- and gain-of-function mutations of beta-catenin in mice. *Genes & development.* 2008;22:2308-2341.

10. Derksen PW, Tjin E, Meijer HP, et al. Illegitimate WNT signaling promotes proliferation of multiple myeloma cells. *Proceedings of the National Academy of Sciences of the United States of America.* 2004;101:6122-6127.

11. Gandhirajan RK, Poll-Wolbeck SJ, Gehrke I, Kreuzer KA. Wnt/beta-catenin/LEF-1 signaling in chronic lymphocytic leukemia (CLL): a target for current and potential therapeutic options. *Current cancer drug targets.* 2010;10:716-727.

12. Coluccia AM, Vacca A, Dunach M, et al. Bcr-Abl stabilizes beta-catenin in chronic myeloid leukemia through its tyrosine phosphorylation. *The EMBO journal.* 2007;26:1456-1466.

13. Serinsoz E, Neusch M, Busche G, Wasielewski R, Kreipe H, Bock O. Aberrant expression of beta-catenin discriminates acute myeloid leukaemia from acute lymphoblastic leukaemia. *British journal of haematology.* 2004;126:313-319.

14. Jauregui MP, Sanchez SR, Ewton AA, et al. The role of beta-catenin in chronic myeloproliferative disorders. *Human pathology.* 2008;39:1454-1458.

15. Geduk A, Atesoglu EB, Tarkun P, et al. The Role of beta-Catenin in Bcr/Abl Negative Myeloproliferative Neoplasms: An Immunohistochemical Study. *Clinical lymphoma, myeloma & leukemia.* 2015;15:785-789.

16. Tefferi A, Thiele J, Orazi A, et al. Proposals and rationale for revision of the World Health Organization diagnostic criteria for polycythemia vera, essential thrombocythemia, and primary myelofibrosis: recommendations from an ad hoc international expert panel. *Blood.* 2007;110:1092-1097.

17. Mesa RA, Verstovsek S, Cervantes F, et al. Primary myelofibrosis (PMF), post polycythemia vera myelofibrosis (post-PV MF), post essential thrombocythemia myelofibrosis (post-ET MF), blast phase PMF (PMF-BP): Consensus on terminology by the international working group for myelofibrosis research and treatment (IWG-MRT). *Leukemia research.* 2007;31:737-740.

18. Thiele J, Kvasnicka HM, Facchetti F, Franco V, van der Walt J, Orazi A. European consensus on grading bone marrow fibrosis and assessment of cellularity. *Haematologica.* 2005;90:1128-1132.
19. Baxter EJ, Scott LM, Campbell PJ, et al. Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. *Lancet.* 2005;365:1054-1061.
20. Jovanovic JV, Ivey A, Vannucchi AM, et al. Establishing optimal quantitative-polymerase chain reaction assays for routine diagnosis and tracking of minimal residual disease in JAK2-V617F-associated myeloproliferative neoplasms: a joint European LeukemiaNet/MPN&MPNr-EuroNet (COST action BM0902) study. *Leukemia.* 2013;27:2032-2039.
21. Liu YC, Lai WC, Chuang KA, et al. Blockade of JAK2 activity suppressed accumulation of beta-catenin in leukemic cells. *Journal of cellular biochemistry.* 2010;111:402-411.
22. Suzuki R, Onizuka M, Kojima M, et al. Infrequent hypermethylation of WIF-1 promoter in BCR/ABL-negative myeloproliferative disorders. *The Tokai journal of experimental and clinical medicine.* 2007;32:131-135.
23. Katoh M. Network of WNT and other regulatory signaling cascades in pluripotent stem cells and cancer stem cells. *Current pharmaceutical biotechnology.* 2011;12:160-170.
24. Katoh M. Networking of WNT, FGF, Notch, BMP, and Hedgehog signaling pathways during carcinogenesis. *Stem cell reviews.* 2007;3:30-38.
25. Zhou S. TGF-beta regulates beta-catenin signaling and osteoblast differentiation in human mesenchymal stem cells. *Journal of cellular biochemistry.* 2011;112:1651-1660.
26. Guo L, Peng W, Tao J, et al. Hydrogen Sulfide Inhibits Transforming Growth Factor-beta1-Induced EMT via Wnt/Catenin Pathway. *PloS one.* 2016;11:e0147018.
27. Lin WH, Xiang LJ, Shi HX, et al. Fibroblast growth factors stimulate hair growth through beta-catenin and Shh expression in C57BL/6 mice. *BioMed research international.* 2015;2015:730139.
28. Takahashi J, Orcholski M, Yuan K, de Jesus Perez V. PDGF-dependent beta-catenin activation is associated with abnormal pulmonary artery smooth muscle cell proliferation in pulmonary arterial hypertension. *FEBS letters.* 2016;590:101-109.
29. Yokoyama Y, Mori S, Hamada Y, et al. Platelet-derived growth factor regulates breast cancer progression via beta-catenin expression. *Pathobiology : journal of immunopathology, molecular and cellular biology.* 2011;78:253-260.
30. Tetsu O, McCormick F. Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature.* 1999;398:422-426.
31. Xu J, Chen Y, Huo D, et al. beta-catenin regulates c-Myc and CDKN1A expression in breast cancer cells. *Molecular carcinogenesis.* 2015.
32. He TC, Sparks AB, Rago C, et al. Identification of c-MYC as a target of the APC pathway. *Science.* 1998;281:1509-1512.
33. Chen S, McLean S, Carter DE, Leask A. The gene expression profile induced by Wnt 3a in NIH 3T3 fibroblasts. *Journal of cell communication and signaling.* 2007;1:175-183.
34. Lam AP, Gottardi CJ. beta-catenin signaling: a novel mediator of fibrosis and potential therapeutic target. *Current opinion in rheumatology.* 2011;23:562-567.
35. Easwaran V, Lee SH, Inge L, et al. beta-Catenin regulates vascular endothelial growth factor expression in colon cancer. *Cancer research.* 2003;63:3145-3153.
36. Levy L, Neuveut C, Renard CA, et al. Transcriptional activation of interleukin-8 by beta-catenin-Tcf4. *The Journal of biological chemistry.* 2002;277:42386-42393.

Table 1. Characteristics of patients with primary (PMF) and secondary myelofibrosis (SMF). Values are presented as proportions or as median and range.

	PMF	SMF
Total number	29	4
Sex	19 / 29 (66%) males 10 / 29 (34%) females	2 / 4 (50%) males 2 / 4 (50%) females
Age	70 (55 – 81) years	71 (57 – 79) years
JAK2 V617F positive	19 / 29 (66%)	2 / 4 (50%)
Hemoglobin level (g/L)	101 (75 – 162)	102 (96 – 135)
WBC (x10^9/L)	10.9 (2.1 – 150.6)	13.1 (6.9 – 22.0)
Platelet count (x10^9/L)	366 (22 - 1491)	438 (276 - 727)
MCV (fL)	86.75 (69.9 – 113.9)	87.5 (69.1 – 118.9)
RDW (%)	19.55 (15.0 - 29.2)	20.1 (18.3 – 25.4)
LDH (U/L)	538.5 (152 – 3400)	795.5 (352 – 1151)
CRP (mg/L)	5.9 (0.5 – 245.0)	8.7 (0.3 – 159.5)
Ferritin (mcg/L)	152 (10 – 10720)	148.5 (16 – 1427)

Figure 1. B-catenin mRNA expression is significantly increased in patients with primary and secondary mylofibrosis in comparison with control group (Kruskal-Wallis one-way analysis of variance, p=0.0002). Relative expression is shown as ΔCT values using abl as the reference gene. ΔCT values are plotted in reverse order for the results to be easier to view and interpret (higher expression is positioned higher in diagram). PMF: primary myelofibrosis, SMF: secondary myelofibrosis; Cont.: control group; n.s.: non significant.