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NKD1 marks intestinal and liver tumors linked to aberrant Wnt signaling

Jitka Stancikova [a,d,1,2], Michaela Krausova [a,2], Michal Kolar [a], Bohumil Fafilek [a], Jiri Svec [a,b], Radislav Sedlacek [a], Magdalena Neroldova [c], Jan Dobes [a], Monika Horazna [a], Lucie Janeckova [a], Martina Vojtechova [a], Martin Oliverius [c], Milan Jirsa [c], Vladimir Korinek [a,*]

[a] Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Videnska 1083142 20 Prague 4, Czech Republic
[b] Second Department of Internal Medicine, Third Faculty of Medicine, Charles University, Prague, Srobarova 50, 100 34 Prague 10, Czech Republic
[c] Institute for Clinical and Experimental Medicine, Videnska 1958/9, 140 21 Prague 4, Czech Republic
[d] Faculty of Science, Charles University in Prague, Albertov 6, 128 43 Praha 2, Czech Republic

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ABSTRACT

The activity of the Wnt pathway undergoes complex regulation to ensure proper functioning of this principal signaling mechanism during development of adult tissues. The regulation may occur at several levels and includes both positive and negative feedback loops. In the present study we employed one of such negative feedback regulators, naked cuticle homolog 1 (Nkd1), to follow the Wnt pathway activity in the intestine and liver and in neoplasia originated in these organs. Using lineage tracing in transgenic mice we localized *Nkd1* mRNA to the bottom parts of the small intestinal crypts and hepatocytes surrounding the central vein of the hepatic lobule. Furthermore, in two mouse models of intestinal tumorigenesis, *Nkd1* expression levels were elevated in tumors when compared to healthy tissue. We utilized a collection of human intestinal polyps and carcinomas to confirm that *NKD1* represents a robust marker of neoplastic growth. In addition, expression analysis of *NKD1* in liver cancer showed that high expression levels of the gene distinguish a subclass of hepatocellular carcinomas related to aberrant Wnt signaling. Finally, our results were confirmed by bioinformatic analysis of large publicly available datasets that included gene expression profiling and high-throughput sequencing data of human colon and liver cancer specimens.

1. Introduction

The Wnt pathway represents one of the principal signaling mechanisms regulating development and tissue homeostasis in metazoan species. In the adult organism, aberrant Wnt signaling is involved in various diseases including cancer (reviewed in [1]). The central mediator of the best studied so-called canonical Wnt pathway is β-catenin, a protein playing a dual role in cell adhesion and signaling (reviewed in [2]). In the absence of an extracellular Wnt stimulus the β-catenin destruction complex, which is composed of casein kinase 1 alpha (CK1α) and glycogen synthase kinase 3 (GSK3), and scaffolding proteins adenomatous polyposis coli (Apc) and axin/conductin, phosphorylates β-catenin on the N-terminal serine/threonine residues. The phosphorylated protein is ubiquitinylated and subsequently degraded by the proteasome. Consequently, in unstimulated cell, the cytoplasmic levels of β-catenin remain low. Interaction of Wnt ligand with the receptor complex comprised of the Frizzled receptor and low-density lipoprotein receptor-related protein 5/6 (LRP5/6) co-receptor disrupts the function of the destruction complex. Subsequently, β-catenin accumulates in the cytoplasm and in the nucleus, where it associates with transcription factors of the lymphoid enhancer-binding factor/T-cell factor (LEF/TCF) family (further referred to as TCFs). Since β-catenin contains a transactivation domain, the TCF-β-catenin complexes act as bipartite transcriptional activators of the Wnt-responsive genes, such as *Axin2*, *c-Myc*, *CD44* or *cyclin D1* (for more comprehensive survey about the Wnt signaling target genes refer to the Wnt signaling home page: http://web.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes).

Abbreviations: APC, adenomatous polyposis coli; BAC, bacterial artificial chromosome; CBC, crypt base columnar; Cp, crossing point; CRC, colorectal carcinoma; CTNNB1, the *β-CATENIN* gene; Dvl, dishevelled; EGFP, enhanced green fluorescent protein; EphB2, ephrin type-B receptor 2; GI, gastrointestinal; GS, glutamine synthetase; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; ISC, intestinal stem cell; ISH, *in situ* hybridization; LacZ, β-galactosidase; Lgr5, leucine-rich repeat containing G protein-coupled receptor 5; Min, multiple intestinal neoplasia; Nkd, naked cuticle homolog; Olfm4, olfactomedin 4; PCA, principal component analysis; qRT-PCR, reverse-transcription quantitative polymerase chain reaction; SD, standard deviation; TA, transit amplifying; TCF, T-cell factor; TCGA, The Cancer Genome Atlas.

* Corresponding author at: Institute of Molecular Genetics AS CR, Videnska 1083, 142 20 Prague 4, Czech Republic. Tel.: +420 241063146; fax: +420 244472282.
E-mail address: korinek@img.cas.cz (V. Korinek).

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[2] These authors contributed equally to this work.

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The intestinal epithelium represents one of the most dynamic tissue in mammalian body (reviewed in [3]). The single-layer epithelium of the small intestine is formed by microscopic projections into the intestinal lumen called villi, and invaginations into underlying mesenchyme, so-called crypts of Lieberkühn. Tissue homeostasis is maintained by intestinal stem cells (ISCs) residing in the bottom part of the crypts. ISCs divide asymmetrically, giving rise to fast-cycling transit amplifying (TA) cells located above the stem cell zone. Descendants of TA cells move up along the crypt/villus axis. At the crypt orifice these cells differentiate to generate specialized cell lineages of the gut that mainly include absorptive enterocytes, mucus-secreting goblet cells, and enteroendocrine cells producing hormones. Differentiated cells continue their movement up to the top of the villus, where they are extruded to the intestinal lumen. The process of epithelial self-renewal is completed in 3–5 days. One exception from this scheme are Paneth cells. The life span of these cells is 6–8 weeks. Furthermore, mature Paneth cells do not migrate from the crypt but stay at the crypt bottom, where they produce antibacterial cryptidins, defensins, and lysozyme (reviewed in [4]). The tissue architecture of the colon reminds the small intestine; however, it does not contain Paneth cells and its surface is flat without the extruding villi. ISCs, alternatively named according to their slender appearance and localization in the lower portion of the crypt as crypt base columnar (CBC) cells, can be recognized by expression of leucine-rich repeat containing G protein-coupled receptor 5 (Lgr5) [5]. Recently, several other markers of ISCs were discovered. These include the tumor necrosis factor receptor family member Troy [6], transcription regulator achaete-scute complex homolog 2 (Ascl2), and an extracellular matrix glycoprotein, olfactomedin 4 (Olfm4) [7]. In addition, CBCs produce high levels of transmembrane ephrin type-B receptor 2 (EphB2), whose expression declines in TA cells, and Paneth cells are EphB2 negative [8].

A number of studies established the Wnt pathway as the main constituent of the signaling network regulating ISCs pluripotency and proliferation (reviewed in [9]). For example, the expression of stem cell markers *Ascl2*, *Lgr5* and *Troy* is governed by the canonical Wnt pathway. Moreover, genetic disruption of genes encoding the Wnt pathway effectors *Tcf4* [10,11] or *β-catenin* [12,13] is associated with demise of intestinal crypts. Interestingly, Wnt/β-catenin signaling plays a key role in the metabolic zonation of the liver (reviewed in [14]). To fulfill its metabolic function, the liver tissue is organized into hepatic lobules, hexagonal-shaped functional units of hepatic parenchyma composed of 15–25 cells. The hepatocytes in the center of the lobule are called perivenous, since they surround a hepatic centrilobular vein. The outer row of lobular hepatocytes line the portal space. These periportal cells are in close contact with the so-called portal triad consisting of the hepatic artery, portal vein and bile duct. Consequently, the periportal hepatocytes receive a mixture of blood originating from both vessels. The portocentrovenular axis of the lobule is the basis for the zonation of the metabolic functions in the adult organ. Cells in different areas of the hepatic lobule are not equal, but they express different genes involved in the metabolism of carbohydrates, xenobiotics, bile production, and detoxification of ammonia. The perivenous hepatocytes contain nuclear β-catenin and they display active Wnt signaling. The Wnt pathway regulates genes encoding enzymes of ammonia detoxication and transport. The genes include *glutamine synthetase* (*GS/Glul*), *ornithine aminotransferase* (*Oat*), and *leukocyte cell-derived chemotaxin-2* (*Lect2*). In contrast, high Apc protein levels in the periportal hepatocytes suppress the Wnt pathway activity. This leads to production of a different set of enzymes mainly participating in ammonia, urea, and carbohydrate metabolism such as *glutaminase 2* (*Gls2*), *arginase 1*, and *phosphoenolpyruvate carboxykinase 1* (*Pck1*) [15].

Aberrant Wnt signaling is found in a multitude of solid tumors, particularly in carcinoma of the colon and rectum [colorectal carcinoma (CRC) [reviewed in [16]]. It is assumed that in the majority of sporadic CRCs the "driver" mutation providing selective advantage to the prospective cancer cell occurs in the *APC* tumor supressor [17,18]. The mutational changes in *APC* lead to production of truncated APC polypeptide that compromises proper functioning of the β-catenin destruction complex (reviewed in [19]). The Wnt pathway might also be activated by inactivating mutations in *AXIN1* [20] or *AXIN2* [21]. In addition, approximately 5% of CRCs contain oncogenic mutations in the *CTNNB1* gene (this gene encodes β-catenin) that alter regulatory amino acid residues at the β-catenin N-terminus and stabilize the protein [22]. The most common type of liver cancer is hepatocellular carcinoma (HCC). The etiology of the disease is heterogeneous and is frequently linked to hepatitis B virus (HBV) or hepatitis C virus (HCV) chronic infection or to alcoholism-related cirrhosis (reviewed in [23]). Recently, several studies performed gene expression profiling and/or high-throughput DNA sequencing to identify the molecular subclasses of HCC or to detect the driver mutations related to liver carcinogenesis [24,25]. Importantly, approximately one-half of HCCs display elevated expression levels of genes activated by Wnt signaling, and oncogenic mutations in the *CTNNB1* gene are found in 20-30% of all HCCs. Analogically to CRC, high levels of stabilized β-catenin and inappropriate transcriptional activation of the TCF-β-catenin target genes is a hallmark of a significant portion of HCCs (reviewed in [26]).

In our previous study we employed chromatin immunoprecipitation in combination with DNA microarray analysis (so-called ChIP-on-chip) to identify genes activated by aberrant Wnt/β-catenin signaling in human CRC cells. One of the genes bound by the TCF4-β-catenin complexes was *naked cuticle homolog 1* (*NKD1*) [6]. Nkd1 is an evolutionarily conserved feedback inhibitor of the Wnt pathway [27]. It interacts with dishevelled (Dvl1) and mediates degradation of this cytoplasmic Wnt signal transducer [28]. Nkd1 reduces aberrant Wnt signaling; nevertheless, in the absence the excessive Wnt signal, its function is less apparent [29,30]. Simultaneous inactivation of both vertebrate paralogs *Nkd1* and *Nkd2* in the mouse leads to only subtle alterations in the morphology of cranial bones and slightly reduced litter size, but the mice are otherwise normal [31]. Based on these and other results mainly obtained in zebrafish [30], Angonin and Van Raay proposed that Nkd1 functions as a "passive antagonist" that acts only when the Wnt signaling levels exceed some threshold [32].

In the present study, we analyzed the *Nkd1* expression pattern in adult mouse gut and liver tissue. Using lineage tracing approaches in transgenic animals we show that *Nkd1* expression is confined to the areas with active Wnt signaling. Furthermore, *Nkd1* transcription was robustly increased in tumors developed in multiple intestinal neoplasia (Min) of mice or in hyperplastic crypts generated by conditional deletion of the *Apc* gene. Using a panel of sporadic tumors of the colon we confirmed that elevated expression of *NKD1* marks neoplastic tissue. In hepatic neoplasia, high *NKD1* mRNA levels, together with upregulation of *AXIN2*, *EPHB2*, and *GS*, distinguish a class of HCCs with deregulated Wnt signaling.

2. Materials and methods

2.1. Experimental animals

$Apc^{+/Min}$ [33,34], Lgr5-EGFP- IRES-CreERT2 [5], Rosa26R [35], and Rosa26R-EYFP [36] mice were purchased from the Jackson Laboratory (Bar Harbor, ME). $Apc^{cKO/cKO}$ [37] mice were obtained from the Mouse Repository (National Cancer Institute, Frederick, MD). Villin-CreERT2 mice [38] were kindly provided by Sylvia Robine (Institut Curie, Paris, France). Animals were housed and handled according to the guidelines approved by the institutional committee. Tamoxifen (Sigma-Aldrich, St. Louis, MO) administration was done by intraperitoneal injection of 1 mg of the compound dissolved in 0.1 ml of corn oil. DNA from tail clippings was used for genotyping. Tissue samples were digested in Proteinase K solution (Thermo Fisher Scientific, Waltham, MA) and precipitated using ethanol; pellets were dissolved in 20 μl of TE buffer (10 mM Tris-HCl, 1 EDTA, pH 8) and 1 μl was used for PCR performed with DreamTaq PCR Master Mix (Thermo Fisher Scientific, Waltham, MA).