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Overexpressed β-catenin Localizes to Plasma Membrane in Respiratory Papillomas

Alexandra V. Lucs[1], **Allan L. Abramson**[1,2], and **Bettie M. Steinberg**[1,2,3]

[1]Feinstein Institute for Medical Research, North Shore-LIJ Health System, Manhasset, NY

[2]Department of Otolaryngology and Communicative Disorders, Hofstra North Shore-LIJ School of Medicine, NY

[3]Department of Molecular Medicine, Hofstra North Shore-LIJ School of Medicine, NY

Recurrent respiratory papillomatosis (RRP), a disease characterized by recurrent tumors of the upper airway, is caused by human papillomavirus (HPV) types 6 and 11 (Doorbar *et al.*, 2012). It has been suggested that the HPV early protein E6 regulates the function of the oncogene β-catenin, including a recent report which shows that HPV16 E6 can induce nuclear localization of β-catenin in a cutaneous mouse model (Bonilla-Delgado *et al.*, 2012). β-catenin's function in the cell is highly dependent on its localization (Brembeck *et al.*, 2006). At the plasma membrane β-catenin mediates the interaction of the adherens junctions, which bind neighboring cells together, and the actin cytoskeleton, which is responsible for organizing internal structures of the cell (Meng and Takeichi, 2009). When β-catenin translocates to the nucleus, it functions as an oncogene because it activates transcription of a number of genes that are important in proliferation and migration. It is not known whether the "low risk" HPVs 6 and 11 induce β-catenin's nuclear localization. We have addressed this question, comparing biopsies of respiratory papillomas to normal tissues from the same patients.

β-catenin localization was determined by immunofluorescence, using E-cadherin as a marker of the plasma membrane and DAPI to mark the nuclei (Figure 1A). There was very strong co-localization of β-catenin with E-cadherin at the plasma membrane, but no evidence of nuclear localization, suggesting that β-catenin was not induced to translocate to the nucleus by HPV 6/11. The localization of β-catenin at the plasma membrane was observed in all papillomas analyzed, regardless of the age of onset or the severity of the papillomatosis. To confirm that β-catenin target genes are not upregulated in papillomas, we reassessed data from our previously published microarray studies that compared mRNA isolated from matched pairs of papillomas and normal laryngeal tissues from 12 RRP patients (DeVoti *et al.*, 2008). The microarrays included probes for 15 of the confirmed

Corresponding author: Alexandra V. Lucs, 350 Community Drive, Manhasset, NY 11030, Telephone: (516) 562-1053, Fax: (516) 562-1022, alucs@nshs.edu.

Conflict of interest

The authors have no conflict of interest to report.

human β-catenin transcriptional targets; myc, cyclin D1, c-jun, uPAR, CD44, ephrin B1, claudin1, vascular endothelial growth factor (VEGF), Met, Endothelin-1, Jagged 1, FGF9, FGF20, Sox9 and Sox17 (Nusse, 2009). Of these, only VEGF was modestly upregulated (data not shown). However, VEGF can be induced by activation of the EFGR, via transcription factors SP1 and AP2 (Pore *et al.*, 2006). Since the EGFR is overexpressed and highly active in papillomas (Johnston *et al.*, 1999), it is likely that VEGF was being induced by this mechanism and not by β-catenin activity.

We noted increased intensity of β-catenin staining in papilloma tissues compared to clinically normal tissues from the same patients, which was confirmed by western blot analysis (Figure 1B). We therefore investigated the mechanism by which HPV 6/11 induced β-catenin overexpression. There was no elevation of β-catenin mRNA levels in papilloma tissues (Figure 1C), suggesting increased protein stability. Two proteins phosphorylate β-catenin, targeting it for degradation: glycogen synthase kinase 3β (GSK-3β) and protein kinase G (PKG) (Heuberger and Birchmeier, 2010). Phosphorylation of GSK-3β was highly elevated in the papillomas (Figure 1D), and phophorylated GSK-3β is inactive. Moreover, PKG levels were generally lower in papillomas (Figure 1E). Thus, HPV 6/11 infection effectively suppresses both mediators of β-catenin degradation, inactivating one kinase and reducing levels of the other.

The second known role for β-catenin is the organization of the actin cytoskeleton. In biopsies of clinically normal tissue from papilloma patients, actin showed clear cortical staining around each cell. In contrast, its distribution in the cells of papilloma biopsies was diffuse and cytoplamic (Figure 2A). Total actin levels do not differ significantly between papilloma cells and normal cells (data not shown). α-catenin, which mediates β-catenin's recruitment of actin to the plasma membrane (Hartsock and Nelson, 2008), was also more cytoplasmically diffuse in papillomas (Figure 2B). We therefore suggest the increased β-catenin in respiratory papillomas results in, or is associated with, it's decoupling from the actin cytoskeleton. Additional work needs to be done to determine the mechanism of this decoupling.

Interestingly, there is a growing body of literature which suggests that the actin cytoskeleton is an active regulator of differentiation of cells in a stratified epithelium. siRNA depletion of ROCK2, a serine/threonine kinase that regulates the cytoskeleton and cell adhesion, suppresses terminal differentiation of keratinocytes (Lock and Hotchin, 2009) Conversely, activation of ROCK2 promotes differentiation (McMullan *et al.*, 2003). More direct evidence comes from a study showing that disruption of actin filaments using latrunculin A results in aberrant expression of differentiation markers (Pedersen *et al.*, 2012). We have previously reported that differentiation is altered in respiratory papillomas (Steinberg *et al.*, 1990), and others have reported that HPV pathogenicity is dependent on altering the differentiation pattern (Longworth and Laimins, 2004). It is clear that the low-risk HPVs are not causing nuclear translocation of β-catenin in respiratory papillomas. Rather, we postulate that they regulate the actin cytoskeleton through manipulation of β-catenin at the cell membrane as a mechanism for altering differentiation.

abbreviations

RRP	recurrent respiratory papillomatosis
HPV	human papillomavirus

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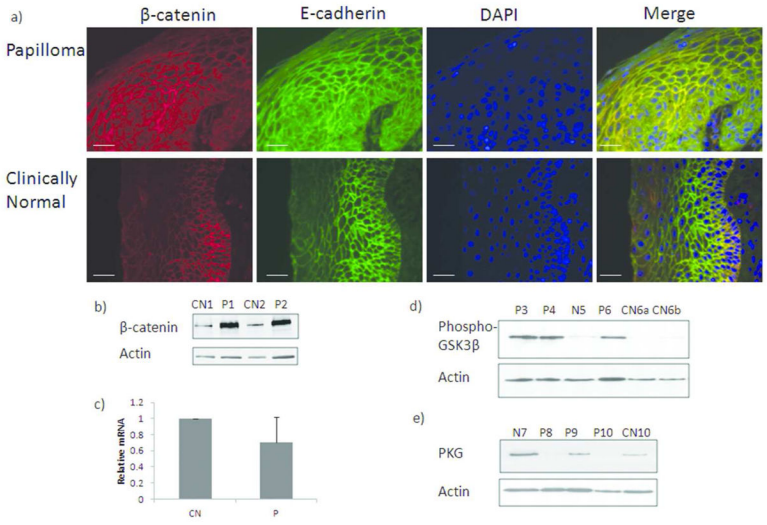

Figure 1. β-catenin localizes to the plasma membrane in respiratory papillomas

Papilloma and clinically normal tissue from the same patient were stained for β-catenin (red) and E-cadherin (green). DAPI (blue) was used to stain the nucleus. The scale bar represents 20 μm. (A) Representative western blots of biopsies of clinically normal tissue (CN) and papilloma tissue (P) from RRP patients. (B) Relative β-catenin mRNA levels, normalized to GAPDH. (C) and (D) western blots of phospho-GSK3β and PKG respectively. N indicates normal samples from a non-RRP patient. Donors of the tissues were numbered sequentially. CN6a and CN6b were clinically normal tissue from two sites in the airway of patient 6. Actin was used as a loading control.

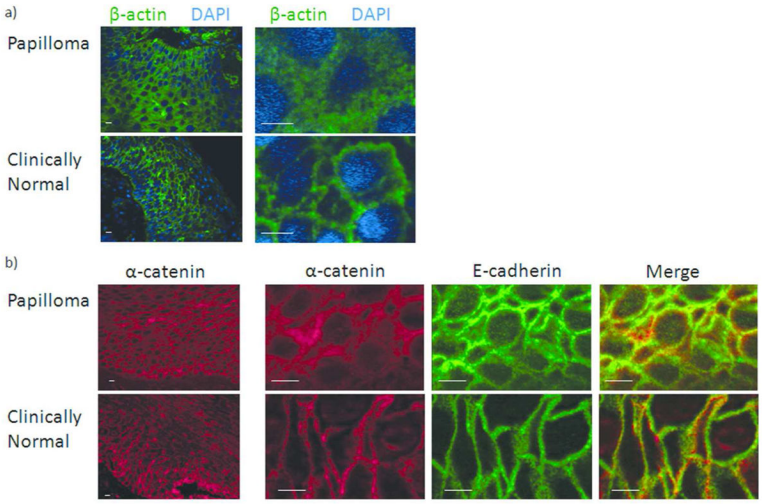

Figure 2. α-catenin and actin are mislocalized in RRP

Immunofluorescent images of papilloma and clinically normal tissue from the same patient stained for β-actin (green) and DAPI to stain the nucleus (blue) (A) or α-catenin (red) and E-cadherin (green) (B). Scale bar represents 5μm.