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Primary Cilia in Tumor Biology: The Primary Cilium as a Therapeutic Target in Cholangiocarcinoma

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Abstract

Cilia are microtubule-based organelles, which are ubiquitously expressed in epithelial cells. Cholangiocytes, the epithelial cells lining the biliary tree, have primary cilia extending from their apical plasma membrane into the ductal lumen, where the cilia function as multisensory organelles transducing environmental cues into the cell interior. The decrease or loss of primary cilia has been described in several malignancies, including cholangiocarcinoma, suggesting that the loss of cilia is a common occurrence in neoplastic transformation. In this short review, we describe the expression of cilia in several cancers, explore the mechanisms and consequences of ciliary loss, and discuss the potential use of the primary cilia as therapeutic targets.

Keywords

Primary cilia; cholangiocarcinoma; ciliotherapy; HDAC6; cholangiocyte; bile duct cancer

1. INTRODUCTION

Cilia are microtubule-based organelles, which are ubiquitously expressed in most cells, including the epithelial cells of the lung, oviduct, breast, prostate, kidney, pancreas, and liver [1]. They are classified according to their microtubule components as 9+2 (motile) and 9+0 (primary) cilia. Multiple motile cilia are present on the epithelial cells lining the airways and reproductive tract. Instead, primary cilia are solitary organelles in many differentiated cell types and function as multi-sensors, detecting and transducing diverse environmental stimuli into the cell interior affecting the cellular behavior. Cholangiocytes, the epithelial cells lining the biliary tree, have primary cilia extending from their apical plasma membrane into the ductal lumen, where the cilia function as mechano-, chemo-, and osmosensors [2–6].

Cilia were once considered vestigial organelles with no physiologically relevant functions, but during the last 20 years it has been observed that mutations in genes required for the

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CONFLICT OF INTEREST

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assembly and/or responsible for the sensory properties of cilia result in diverse human disorders, now referred as ciliopathies. Therefore, cilia have become the subject of intense investigation, resulting in the conclusion that cilia are important to normal cell function [6–9]. Thus, it is essential that the architecture and function of these cellular structures are accurately regulated and maintained.

Cholangiocarcinoma (CCA), is a malignancy thought to be derived from cholangiocytes. CCA can be divided in intrahepatic and extrahepatic, with the last being further subdivided in perihilar and distal tumors. Hepatobiliary malignancies account for 13% of the annual cancer-related deaths worldwide and for 3% in the United States [10–12]. Importantly, more than 2,500 new cases of CCA are diagnosed in the United States each year, and in the last four decades, the incidence rates of intrahepatic CCA have increased by 165%. CCA is usually diagnosed late and it is a lethal cancer with very limited therapeutic options [13–17]. Therefore, it is imperative to identify novel targets that can lead to new therapeutic strategies for this devastating disease.

2. PRIMARY CILIA FATE IN CANCER

Many of the abnormalities found in cells involved in ciliopathies, *i.e.* loss of response to environmental signals, increased cell proliferation, cell polarity alterations, and abnormal extracellular matrix control leading to fibrosis, are also present in tumor cells [18]. Since cilia function as a hub for the activation of the canonical pathway of Hedgehog (Hh) signaling and other growth factors, it was first hypothesized that primary cilia could stimulate or enhance tumor development [19–21]. Indeed, two reports support that scenario; in medulloblastoma and basal cell carcinoma, smoothened-dependent tumors require the presence of primary cilia to develop [22, 23].

In contrast, in several others malignancies, primary cilia are decreased or lost in the tumor cells (Table 1), suggesting that the loss of cilia is a common occurrence in neoplastic transformation. However, these observations have been received with certain skepticism, since primary cilia is intimately linked to the cell cycle, and is expected that in highly proliferative cells, primary cilia would be absent. However, several studies showed that the absence of cilia is independent on the cell's proliferation status, suggesting that intrinsic mechanisms exist in cancer cells to avoid ciliogenesis [24–26].

The mechanisms of ciliary loss in different tumors remain to be elucidated, but some insight has recently emerged. In cholangiocarcinoma (CCA), ciliary expression was found to be decreased, both *in vivo* and *in vitro*, when human samples and human cell lines were assessed [27, 28]. The mechanisms underlying the reduction of cilia in CCA appears to be linked to the overexpression of histone deacetylase 6 (HDAC6), an enzyme that deacetylates tubulin in the ciliary axoneme inducing their resorption [27]. In pancreatic adenocarcinoma (PDAC), the absence of cilia is independent of ongoing proliferation and the loss of cilia can be reversed by inhibiting Kras pathways [24]. Interestingly, Kras activates HDAC6, which may link the loss of primary cilia to similar mechanisms in CCA and PDAC [29].

Ovarian cancer cells also display a significantly reduced number of primary cilia by a mechanism linked to the overexpression and persistent localization of Aurora A kinase (AURA) to the ciliary basal body. The knockdown of AURA partially induced ciliary restoration [30]. Even though AURA is a known activator of HDAC6, increased acetylation of the microtubules was not observed. This can potentially be explained by an alternative mechanism of deciliation or, a site-specific inactivation of HDAC6, which may induce the hyperacetylation only in the nascent ciliary axoneme, a scenario that would be difficult to detect by western blot analysis of total cell lysates [30].

Reduced ciliary expression has been also reported in renal cell carcinomas [25, 31–33]. In the most characterized tumor (*i.e.*, clear cell renal carcinoma), the loss of cilia is mediated by the downregulation or dysfunction of the von Hippel-Lindau (VHL) tumor suppressor gene. VHL binds and stabilizes microtubules, and its inactivation induces Hef1 and AuroraA by a mechanism involving β -catenin, leading to activation of HDAC6, once more suggesting an important role of HDAC6 in ciliary loss in tumors of different origins [32].

Aberrant ciliogenesis is also found in cell lines derived from astrocytoma/glioblastoma, but more recent studies using human tissues suggest that cilia may be present, although reduced in frequency by 8–25% [34–36]. Primary cultures derived from these tumors failed to demonstrate increased ciliogenesis after serum starvation, suggesting an intrinsic mechanism blocking ciliary formation, possibly involving the upregulation of the cell cycle-related kinase (CCRK) which acts through two of its substrates, the intestinal cell kinase (ICK) and MAK (male germ cell-associated kinase) kinases that control ciliary length [35].

In a recent manuscript, Rocha *et al.* described another interesting example of the role of cilia in tumorigenesis. They demonstrated that an additional posttranscriptional modification of the ciliary axoneme, *i.e.*, tubulin glycylation, regulates the stability of primary cilia. The absence of TTLL3, a tubulin glycine ligase, leads to a reduced number of cilia in colon epithelium, increased cell proliferation, and amplification of tumor development in mice [37]. Interestingly, analysis of tumors from colorectal cancer patients confirmed that a decreased level of TTLL3 is a risk factor for the development of the disease, suggesting potential as a prognostic marker for colon cancer [37].

Other tumors presenting loss or decreased cilia expression include prostate, breast, melanoma, chondrosarcoma/enchondromas, and rhabdomyosarcoma, but the mechanisms involved in ciliary loss in these cancers remain to be elucidated [26, 38–43].

Finally, it was recently reported in a human renal proximal tubular epithelial cell line that the loss of primary cilia could also be the result of exposure to chemical carcinogens [44]. This observation may be particularly relevant to cholangiocyte cilia that are exposed to xenobiotics, bile acids, and biliary components, which may represent an additional mechanism for ciliary loss in cholangiocarcinoma.

As mentioned before, the examples of medulloblastoma and basal cell carcinoma indicate that cilia may act as a tumor suppressor or promoter dependent on the oncogenic context, especially for Hh driven tumors where active mutations upstream or downstream of the primary cilia will dictate its role as promoter or suppressor, respectively [22, 23, 45].

3. WHAT ARE THE CONSEQUENCES OF CILIARY LOSS?

The multisensory functions of primary cilia depend on the expression of specific receptors and channels in the ciliary membrane as well as the complex system accounting for ciliary biogenesis, the intraflagellar transport (IFT) system, and the pathways responsible for the stability of microtubules. A plethora of receptor signaling components and ion channels are heavily enriched in the primary cilium, including polycystin-1 and -2, fibrocystin, transient receptor potential cation channel, subfamily V, member 4 (TRPV4), purinergic receptor P2Ys, the G protein-coupled bile acid receptor TGR5, Hedgehog and Wnt signaling components, Platelet-derived growth factor receptor (PDGF-R), and transforming growth factor beta receptor (TGF β), among others [2–4, 46–54].

In epithelial tumors that are characterized by the loss of primary cilia, disturbed signaling mediated by the cilium could lead to promotion of proliferation and perhaps migration and invasion. For instance, cholangiocyte primary cilia express the bile acid receptor TGR5, that when activated inhibits cell proliferation by decreasing cAMP levels in the cell [46, 55, 56]. Interestingly, when cilia are not present, TGR5 localizes mainly to the plasma membrane and its stimulation produces the opposite effect, *i.e.* increased proliferation and cAMP levels. The mechanism underlying this paradoxical effect seems to be the differential coupling of TGR5 with G α s or G α i in the plasma membrane and the cilium, respectively [46].

On the other hand, cholangiocyte cilia also express purinergic receptors, like P2Y₁₂, that are activated by nucleotides in bile inducing a decrease in cAMP leading to decreased proliferation [4]. Another important signaling pathway, the Hedgehog pathway, involves primary cilia, and depending on the oncogenic context, the loss of cilia can induce activation of this pathway [22, 23].

Therefore, it is plausible that the multisensory functions of primary cilia act as a brake on cell proliferation, migration and invasion, and their loss during tumorigenesis gives an advantage to these cancer cells to freely proliferate. Supporting this hypothesis, recent observation indicates that cilia inhibit cell cycle re-entry [57, 58]. In fact, when normal cholangiocytes are deciliated by chemical or molecular approaches, cells show a malignant-like phenotype including increased proliferation, invasion and anchorage independent growth, together with the activation of MAPK and Hh signaling pathways, both important pathways involved in CCA progression [27].

Several issues remain to be elucidated: i) How is the interplay between the cilium and cell cycle regulated? It is clear that this is a two-way highway and the dysregulation of one of them affects the other and vice-versa; ii) are the intracellular signals generated by receptors located in the primary cilium different if the cilia are absent and the receptors are then mislocalized to the plasma membrane? This question needs to be addressed specifically for each receptor of interest. For example, as mentioned above, the G-coupled receptor TGR5, a bile acid receptor, has distinct functions dependent on its localization. When in the cilium, it is coupled to G α i and produces a decrease in intracellular levels of cAMP. On the other hand, when located at the plasma membrane, it couples with G α s and its activation induces

cAMP levels and proliferation of the cholangiocytes [46]; iii) at what phase during tumor development are cilia lost? This is an important question that needs to be addressed for each particular cancer, and may help to further clarify the role of this organelle in tumor development. Two studies have shown that primary cilia are lost early in the development of pancreatic and breast cancer, and that the absence of cilia is associated with the prognosis of the disease [41, 59]. In PDAC cases, cilia were identified in one-quarter of cases, and its presence was associated with poor prognosis, while in breast cancer the loss of cilia correlated with higher grade of invasiveness and occurred early in cancer development [41, 59].

3. CILIOETHERAPIES

Accumulating data from solid tumors derived from epithelial cells where the cilium is facing a luminal side, strongly suggest that this organelle functions as a tumor suppressor. Therefore, the mechanisms that cancer cells develop to inhibit ciliogenesis give them a selective advantage to proliferate. Thus, therapeutic approaches oriented to the restoration of primary cilia expression, ciliotherapies, can be hypothesized to induce the re-differentiation of tumor cells to a more normal-like phenotype that might reduce tumor growth or even initiate cell death in the often oxygen and nutrients deprived environment that a transformed cell uses to growth.

Work focused in the classic ciliopathy, polycystic kidney and liver disease (PKD/PLD), showed that the phenotype of cystic cholangiocytes included loss of the sensory function and a hyperproliferative phenotype dependent on MAPK [60]. Importantly, our previous work also showed that the replication of one of the effects of the environmental stimuli detected by cilia, *i.e.* the increase in intracellular calcium by the osmosensor TRPV4, decreased both, MAPK activity and cell proliferation [3, 61]. Since the loss of primary cilia induces a malignant-like phenotype in normal cholangiocytes, the restoration of cilia in tumor cells may represent a potential therapeutic approach [27].

While the approach to the restoration of primary cilia may well vary with each type of tumor, activation of HDAC6 appears to be a common event in those cancers where the mechanisms have been explored, including cholangiocarcinoma, pancreatic, renal, and likely ovarian cancer [24, 27, 30, 32]. HDAC6 is a member of the histone deacetylase family of proteins that is localized exclusively in the cytoplasm, and functions as a tubulin deacetylase [62]. Interestingly, acetylated- α -tubulin is one of the main structural components of the primary cilium, and its deacetylation is required for ciliary disassembly [29]. Our group successfully targeted HDAC6, *in vitro* using cell lines and in animal studies using an orthotopic model of cholangiocarcinoma. In the *in vitro* experiments, downregulation of HDAC6 by shRNAs or the pharmacological inhibition by tubastatin-A induced a restoration of primary cilia expression and a reduction of cell proliferation and anchorage independent growth, together with a decrease in MAPK and Hh signaling pathways activities. Importantly, when CCA cell lines were stably transfected with specific shRNAs to one of the components of the IFT apparatus, in order to prevent the restoration of cilia, the effect of tubastatin-A on proliferation and anchorage independent growth was inhibited. Taken together, these observations suggest that the restoration of cilia is the main driver of

HDAC6-inhibition effects in cholangiocarcinoma cells. *In vivo*, the inhibition of HDAC6 induced a decrease in tumor growth accompanied with a partial restoration of primary cilia in tumor cells [27].

Ciliotherapies may be important also in PKD; we recently showed that HDAC6 inhibition by ACY-1215, a more specific and potent HDAC6 inhibitor, reduces cell proliferation *in vitro* and cyst growth both *in vitro* and in an animal model of the disease, but the role of cilia in this response remain to be investigated [63]. In a recent study, the concept of ciliotherapy was successfully applied by using fenoldopam to enhance cilia function by extending the length of cilia [64]. Kathem *et al.* showed both, *in vitro* and *in vivo* that fenoldopam increases ciliary length in vascular endothelial cells, which have been shown to have abnormal sensory functions in PKD. As a consequence, the treatment induced increased nitric oxide serum levels and reduced blood pressure in a PKD mouse model, suggesting that this approach may be important to address the hypertension associated with PKD. Furthermore, a clinical study indicated that in fact, this therapy produces an overall reduction in the arterial pressure in PKD patients [64].

The use of HDAC6 specific inhibitors to restore or increase ciliary stability has several advantages, since HDAC6 is one of the only members of the histone deacetylase family that does not interact with histones, avoiding the myriad of side effects caused by the use of HDAC pan-inhibitors [65]. Furthermore, the rapidly evolving field of HDAC inhibitors promises the development of more potent and specific molecules like the recently developed ACY-1215 and ACY-738 [63, 66]. The fact that HDAC6 knock out mice are viable and develop normally, suggests that HDAC6 targeting may have minimal adverse effects [63, 67]. Therefore, the restoration of primary cilia by specific HDAC6 inhibition may be a potential therapeutic target for cholangiocarcinoma [27].

If loss of the primary cilium proves to be an integral part of the complex and diverse mechanisms of malignant cell transformation, ciliotherapy might have potential value not only for treatment of tumors but also for cancer prevention.

CONCLUSION

Several evidences from different types of tumors have shown that primary cilia may play an important role in tumorigenesis and tumor progression by acting as a tumor suppressor organelle. The field still needs to address if the loss of cilia precedes and contributes to cell transformation or, if that loss is a secondary effect of cell transformation that in turn may enhance the growth abilities of the transformed cell [18]. Whatever the case, it will certainly be tissue specific, with the restoration of cilia holding great promise in becoming a potential therapeutic approach.

The mechanisms by which cilia act as a tumor suppressor remain to be elucidated. Beyond the known ability of cilia to participate in the generation of the Hh signaling repressor Gli3 [22, 23], other mechanisms needs to be identified. Our group is actively investigating how the multisensory functions of cholangiocyte primary cilia regulate important pathways involved in carcinogenesis, like MAPK and p53. The increasing interest in the role of cilia in

tumor biology warrants larger studies to assess whether the level of expression of cilia correlates with the different stages or subtypes of CCA and if there is any prognostic value. Furthermore, in the emerging era of individualized medicine, this information will be critical to consider a ciliotherapy as potential treatment.

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Table 1

Primary cilia expression in different malignancies.

Cancer	Cilia	Mechanism	Reference
<i>Pancreas</i>	Lost	Excessive activation of Kras pathways	[24, 59]
<i>Breast</i>	Lost	Unknown	[39–41]
<i>Melanoma</i>	Lost	Unknown	[26]
<i>Renal Cell Carcinoma</i>	Lost	Aurora kinase A overexpression	[25, 31–33]
<i>Cholangiocarcinoma</i>	Lost	HDAC6 overexpression	[27, 28]
<i>Glioblastoma</i>	Lost	CCRK upregulation	[34–36]
<i>Prostate</i>	Lost	Unknown	[38]
<i>Ovarian</i>	Lost	Aurora kinase A overexpression	[30]
<i>Chondrosarcoma/Enchondromas</i>	Lost	Unknown	[42]
<i>Rhabdomyosarcoma</i>	Lost	Unknown	[43]
<i>Colon</i>	Lost	TTL3 downregulation	[37]
<i>Medulloblastoma</i>	Present		[22, 45]
<i>Basal cell</i>	Present		[23]
<i>Gastric Gastrointestinal Stromal Tumours</i>	Present		[68]