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ORIGINAL ARTICLE

Sonic Hedgehog Protein is Frequently Up-Regulated in Pancreatic Cancer Compared to Colorectal Cancer

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Abstract

Sonic hedgehog (SHH) is a secreted protein which functions in autocrine or paracrine fashion on target cells to activate hedgehog (HH) signalling cascade responsible for growth and proliferation. This study is an attempt to understand the expression dynamics of SHH protein in colon, rectal and pancreatic cancers. Protein expression of SHH was studied by Western Blotting in the histologically confirmed colon, rectum and pancreatic cancer tissue samples along with their adjacent normal tissues. Only 31.4% (11 of 35) and 26.9% (7 of 26) of colon and rectal cancer cases respectively showed an increase in SHH expression in tumours compared to 72.7% (24 of 33) of the pancreatic cancer cases when compared with their adjacent normal tissues. Our results suggest that SHH may have a strong role in the predisposition of Pancreatic cancer and could possibly be used as a diagnostic or prognostic biomarker.

Keywords Sonic hedgehog · Hedgehog signalling pathway · Protein expression · Western blotting · Colorectal cancer · Pancreatic cancer

Introduction

Colorectal and Pancreatic cancers, both cancers of the gastrointestinal tract are on the rise. Majority of colorectal and pancreatic cancers are adenocarcinomas. According to GLABACON 2012 database colorectal cancer has worldwide morbidity and mortality and is the 4th leading cause of cancer-related deaths in the western world with 700,000 deaths in 2012 [1]. Pancreatic cancer, a deadly lethal malignant neoplasm is the 7th leading cause of death worldwide, more common in developed countries of the world with more than 338,000 death in 2012 [2, 3]. Data compiled by American Cancer Society for the year 2017 estimated 135,430 and 53,670 new colorectal and pancreatic cancer cases and 50,260 and 43,090 deaths respectively due to these cancers [4]. Aberrant activation of many oncogenic signalling mechanisms like Mitogen-activated protein kinase, TGFβ, Phosphatidyl Inositol 3-kinase and Wnt signalling have been linked to Colorectal and Pancreatic cancers [5–7] but not much has been achieved with regard to early diagnosis and treatment in a previous decade. Hedgehog signalling pathway has emerged as a new signalling pathway to have essential roles in embryogenesis and carcinogenesis.

Hedgehog (HH) signalling pathway is known to play a key role in various developmental processes in Drosophila as well as in vertebrates [8]. It has crucial functions in embryonic development, maintenance of adult tissues and oncogenesis. The three ligands sonic hedgehog (SHH), Desert hedgehog (DHH) and Indian hedgehog (IHH) which function in autocrine, paracrine or juxtacrine fashion mediate the action of HH pathway [9]. They are synthesised as 45 kDa protein which undergoes numerous post-translational modifications to form biologically active 19 KDa proteins [10].

The sonic hedgehog signalling cascade is activated when sonic hedgehog binds to Patched 1 (PTCH1), a 12 pass transmembrane protein. This binding releases the inhibitory effect on a protein named Smoothened (a 7 pass GPCR like protein) and as a result of activation of smoothened hedgehog signal is transmitted via several transcription factors. These induce

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transcription of genes responsible for cell growth, differentiation and proliferation. In the absence of hedgehog ligand binding, PTCH1 inhibits Smoothened and no signal is transmitted and the pathway is switched off [11].

Dysregulation of the hedgehog pathway has been highly implicated in birth defects and tumorigenesis [5–7]. Aberrant Hedgehog signalling has been reported in Medulloblastomas, Basal Cell Carcinoma, Rhabdomyosarcomas, and Pancreatic cancer [12–15]. SHH overexpression has been found to promote tumour growth and metastasis in Pancreatic cancer [16] and higher levels of SHH are associated with poor survival and poor prognosis in breast cancers [17]. Moreover, the stronger SHH expression is also known to be involved in the early phase of Gastric cancers [18]. Active hedgehog signalling has also been reported in Prostate, Lung, Liver, Thyroid, Bladder, Ovary and Colon Cancers [19–25].

In Kashmir Valley, the incidence of Colorectal cancer has shown an alarming increase and ranks as the 4th most common cancer [26]. In spite of advances in treatment, the mortality rate is still very high. On the other hand, Pancreatic cancer in Kashmir has also shown a tremendous increase in the past few years [26]. It is a dreadful malignancy with very poor prognosis and survival. Most patients when diagnosed are in the late stage of disease and surgically unresectable and therefore there is an imperative need to identify markers that would aid in early diagnosis and improve patient survival.

Some studies have reported aberrant activation of the SHH pathway in Pancreatic cancer. However, its role in Colorectal cancer is indecisive. Hence, we conceived this study to investigate the expression of SHH protein in Colorectal and Pancreatic cancers to access the involvement of SHH signalling pathway in the genesis of Colorectal and Pancreatic cancers.

Materials and Methods

Patients and Samples

Overall thirty-five ($n = 35$) Colon, twenty-six ($n = 26$) Rectal and thirty-three ($n = 33$) Pancreatic cancer tissues and their histologically confirmed adjacent normal tissues were included in the present study. The study was conducted in the Department of Clinical Biochemistry in association with the Department of General Surgery and Surgical Gastroenterology, SKIMS, Srinagar, India. Samples collected between 2015 and 2017 were included in the study. Tissues collected after surgery were immediately snap frozen in liquid nitrogen before further processing. All patients had the first-time diagnosis and did not receive any chemo or radiotherapy.

Protein Extraction

For protein extraction tissue samples were washed 3 times with ice-cold phosphate buffer saline (PBS) by centrifuging at 7000 rpm for 5 min. The pellet was then homogenized with the freshly prepared NP-40 lysis buffer (20 mM Tris, 150 mM NaCl, 0.5% Sodium deoxycholate, and 1 mM EDTA, 0.1% SDS, 1% Nonidet P-40) and protease inhibitor cocktail (1 mM PMSF, 5-10 mM NaF and PIC). Samples were vortexed and incubated on ice for 1-h following which samples were centrifuged at 10000 rpm for 20 min and the supernatant was collected to obtain protein extract. The protein concentration was then determined spectrophotometrically at 595 nm using the Bradford assay.

Antibodies

Rabbit monoclonal antibody against SHH (*Cell Signalling technology; USA*) was used at 1:1000 dilution. For loading control mouse monoclonal antibody against beta-actin *(Cell Signalling technology; USA)* was used at 1:1000 dilution. For final detection of protein bands, Florescence tagged secondary antibody (anti-rabbit IR Dye 800; dilution of 1:10000) and anti-mouse IR dye 680 dilutions of 1:20000) from *LI-COR Biosciences; the USA* was used.

Western Blotting

Total protein extracts (40 μg) from normal and tumour tissues were resolved on 12% SDS-PAGE and transferred to PVDF membrane (Millipore; USA) using a semi-dry transfer method in accordance with manufacturer protocol (Hoefer; USA). The PVDF membrane was then blocked with 3% BSA in PBS for 1 h, after which the membrane was probed with primary antibodies overnight. Washing was done with PBS tween 20 (PBST). For final detection membrane was probed with secondary antibodies. Fluorescence was detected using Odyssey infrared detection system (*LI-COR Biosciences, USA*). For quantification of western blots, densitometric analysis was performed using Image J software (*NIH Maryland USA*).

Statistical Analysis

All experiments were performed in triplicate, and results were calculated as mean ± SD. Fold change in protein expression was calculated by densitometry using Image J software. *(NIH Maryland USA).*

Results

SHH Expression in Cancers of Colon and Rectum

Out of 61 Colorectal cancer tissues, 35 were Colon adenocarcinomas, 26 cases were confirmed as Rectal adenocarcinomas (Table 1). Expression of SHH protein was evaluated in lysates extracted from cancerous tissue samples of Colon and Rectum along with their histologically confirmed adjacent normal tissues. It was observed that SHH protein was expressed in all tumours and their adjacent normal tissues. In most of the cases, the intensity of SHH expression in tumour tissues was comparable to that of their adjacent normal tissues. Out of the 35 Colon carcinoma cases studied, only 11 (31.4%) showed one to a two-fold increase in the expression of SHH protein when compared to their adjacent normal tissues. Among the 11 samples which showed overexpression of SHH protein 07 (63.6%) were well differentiated (WD) 02 (18.1%) were moderately differentiated (MD) and 02 (18.1%) were poorly differentiated (PD) cancers (Fig. 1). Out of the 26 Rectal cancer cases studied, only 07 [05; WD (71.4%), 01; MD (14.28%) and 01; PD (14.28%)] showed one to two-fold increase in SHH expression in tumours as compared to their adjacent normal tissues which accumulate to 26.9% of total rectal cancer cases (Fig. 2). These results were irrespective of the age and sex of subjects involved.

SHH Expression in Pancreatic Cancer

Out of 33 Pancreatic cancer tissues, 28 were confirmed as Pancreatic Ductal Adenocarcinoma (PDAC), 03 were Pancreatic Neuroendocrine Tumours (NEUT) and 02 were Pancreatic Mucinous Neoplasms (MUCN). (Table 1). When expression of SHH was compared between Pancreatic cancer and their respective adjacent normal tissues we found SHH was significantly overexpressed in 72.7% (24/33) of the cases with 4 to 6 fold in tumour tissues when compared to their adjacent normal tissues. Out of total 33 Pancreatic cancer tissues (28; PDAC, 03; NEUT, 02; MUCN) overexpression of SHH protein was found in 24 (72.7%) tumour tissues (20; PDAC, 03; NEUT, 01; MUCN) when compared to their adjacent normal tissues (Table 1; Fig. 3).

Discussion

SHH signalling has important roles in cellular growth and differentiation. SHH, a key component of the pathway on activation transports signals downstream to activate nuclear transcription factors (GLI) responsible for proliferation [27]. SHH signalling plays a key role in embryonic development, maintenance of normal adult tissues and tumorigenesis. SHH over-activation has also previously been linked gastrointestinal cancers but conclusive information is lacking [28].

In this study, we investigated the protein expression of SHH in Colorectal and Pancreatic cancer to build up our understanding of the identification of cancers that could be targeted by SHH inhibitors. The role of SHH activation in Colorectal cancer is inconclusive with many studies presenting contradictory results. A study by Mazumdar et al. (2011) demonstrated that HH signalling drives cellular survival of Colon carcinoma cells [29]. Another study by Varnat et al.

Table 1 Characteristics of colorectal and pancreatic cancer patients

Characteristics	Males		Females		Percent SHH over expression
Total ($n = 94$)	n	%	n	%	–
Gender	52	55.31	42	44.68	–
Mean Age	58.44 ± 14.14		53.45 ± 13.97		–
Colon adenocarcinoma	21	40.38	14	33.33	31.4%
Well differentiated	8	15.38	8	19.04	38%
MD differentiated	10	19.23	4	9.5	15%
PD differentiated	3	5.76	2	4.76	20%
Rectal adenocarcinoma	9	17.30	17	40.47	26.9%
Well differentiated	7	13.46	10	23.80	30%
Moderately differentiated	1	1.92	5	11.90	16.6%
Poorly differentiated	1	1.92	2	4.76	33%
Pancreatic cancer	22	42.30	11	26.1	72.7%
Pancreatic adenocarcinoma	19	36.53	9	21.42	72%
Mucinous neoplasms	0	0	2	4.76	50%
Neuroendocrine tumors	3	5.76	0	0	100%

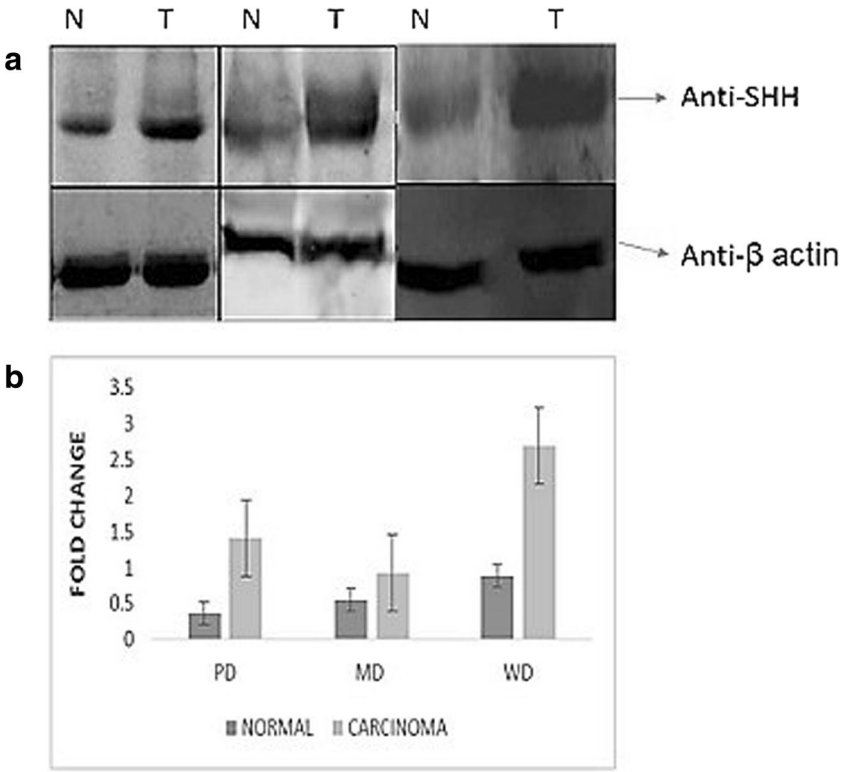

Fig. 1 **a** Immunoblot showing the expression of SHH protein in poorly differentiated (PD) moderately differentiated (MD) and well differentiated (WD) Colon carcinoma cases and their adjacent normals (N). **b** Bar chart comparing the fold change in expression levels of SHH protein

(2009) showed active HH signalling is essential for tumour growth recurrence metastasis and stem cell survival and expansion [25] while as in contrast some of the studies have established that SHH is downregulated in colon cancer and involved in differentiation of normal colonic tissues [30].

In cancers of Colon and Rectum for many samples, we could not detect a considerable difference in SHH protein levels between a tumour and adjacent normal tissues. Our results are in line with those reported by Chatel et al. (2007) who proposed that aberrant activation of SHH signalling is not common in Colorectal cancer cell lines [31]. Douard et al. (2006) established SHH mRNA is overexpressed in 86% of Colorectal adenocarcinomas [32]. In our study, we observed a much less increase of SHH protein in Colon adenocarcinoma

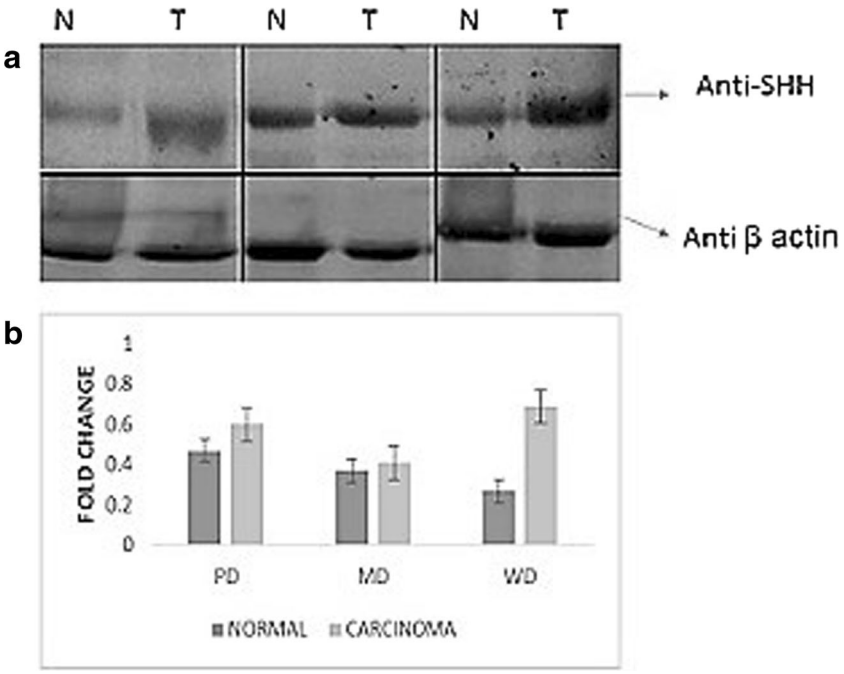

Fig. 2 **a** Immunoblot showing the expression of SHH protein in poorly differentiated (PD) moderately differentiated (MD) and well differentiated (WD) Rectum carcinoma cases and their adjacent normals (N). **b** Bar chart comparing the fold change in expression levels of SHH protein

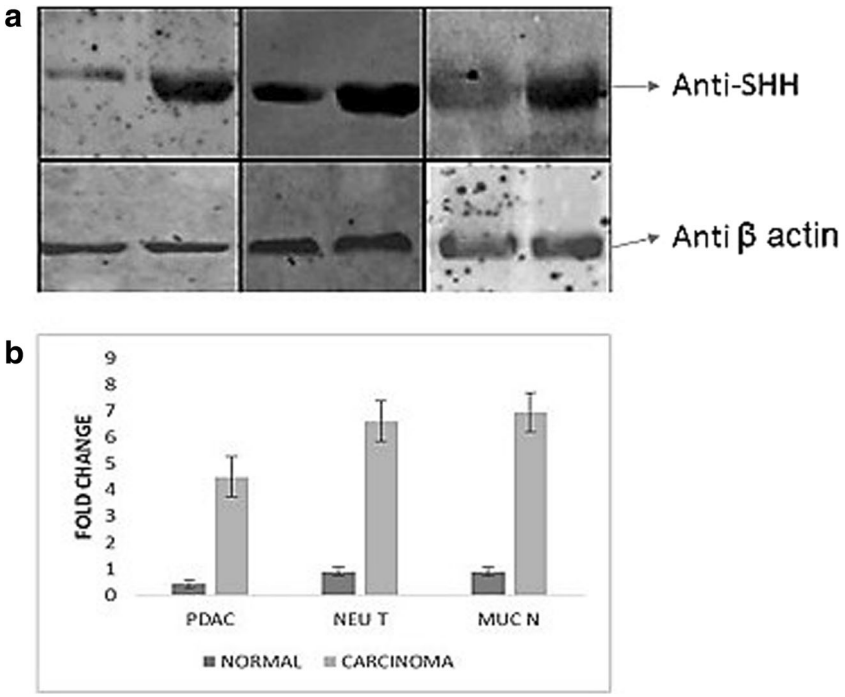

Fig. 3 **a** Immunoblot showing the expression of SHH protein in Pancreatic ductal adenocarcinoma (PDAC) a Neuroendocrine tumour (NEUT) and mucinous neoplasms (MUCN) cases and their adjacent normals (N). **b** Bar chart comparing the fold change in expression levels of SHH protein

(31%) and Rectal adenocarcinoma (26.9%). This difference in the frequency of protein expression may be due to differences in the technique used for protein estimation and due to sample size bias.

In Pancreatic cancer, our results indicate overexpression of SHH protein (72.7%) which is in consistency with previous reports [15]. Not a single study till date has been reported suggesting the downregulation of SHH protein in Pancreatic cancer. A study by Thayer et al. (2003) showed that SHH is overexpressed in 70% of PDAC [15] Another study reported that SHH activation is a common event in Pancreatic cancer. Another study by Ohuchida et al. (2006) suggested that SHH is an early developmental marker of intraductal papillary mucinous neoplasm of Pancreas [33]. Marechal et al. (2015) established that SHH is a prognostic biomarker in Pancreatic Ductal Adenocarcinoma [34] In one more study, the authors proposed that HH signalling is involved in cell proliferation, metastasis and resistance to therapy in Pancreatic cancer [35].

Although the SHH protein was overexpressed in both Colorectal and Pancreatic tumour tissues the number of cases showing overexpression was more in case of Pancreatic cancer (72.7%) as compared to the Colon (31.4%) and Rectal cancer (26.9%). Also, the relative fold change was more in case of Pancreatic tumour tissues (4–6 fold) with respect to Colorectal tumour tissues (1–2 fold) when compared to their adjacent normal tissues. Although, increased expression of SHH has also been reported in Breast, Esophageal, Gastric and Pancreatic cancer by previous studies [17, 36–38] but to our knowledge this study is the first attempt towards understanding expression dynamics and comparing the fold increase of SHH protein in Colon, Rectal and Pancreatic cancer cases and their adjacent normal tissues.

Conclusion

In Conclusion, our observations suggest that SHH is up-regulated in Pancreatic cancer as compared to Colon and Rectal cancer. Since our results proposed SHH is considerably up-regulated in Pancreatic cancer we suggest aberrant activation of SHH has a substantial role in the development and progression of Pancreatic cancer. Overall our results point towards the involvement of SHH as a prognostic or diagnostic biomarker in Pancreatic cancer. Although the results are convincing we still propose that the current study needs more confirmation and validation with an increased sample size.

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Compliance with Ethical Standards

Conflicts of Interest The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Informed Consent All the patients were informed about the study and tissue samples were obtained after taking written consent from patients.

Ethical Approval The study was approved by the Ethical Clearance Committee of SKIMS (SIMS 1131/IEC-SKIMS/2015–183).

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