Clin Transl Oncol (2012) 14:138-142
DOI 10.1007/s12094-012-0773-7

RESEARCH ARTICLES

Examination of Smad2 and Smad4 copy-number variations in skin cancers

Yong Shao* · Jie Zhang* · Richu Zhang · Jun Wan · Wei Zhang§ · Bo Yu§

Received: 9 February 2011 / Accepted: 21 May 2011

Abstract
Background Smad2 and Smad4 transcription factors were identified as the signalling mediators of transforming growth factor ß (TGF ß) pathway. Copy number variations (CNVs) have been discovered to have phenotypic consequences and be associated with various types of cancers. CNVs of Smad2 and Smad4 were found to be associated with cancer pathogenesis in the recent array-based study. However, no such study has been performed in skin cancer yet. In this study, we aim to examine the CNVs of Smad2 and Smad4 in skin samples.
Methods A total of 195 paired samples including basal cell carcinoma (BCC), squamous cell carcinoma (SCC) and actinic keratosis (AK) were included. Real-time PCR was used for the quantification of Smad2 and Smad4 copy numbers.
Results CNVs of Smad2 showed statistical differences between cancer samples (both SCC and BCC) and normal tissues ($p<0.05$). For Smad4, statistical difference was observed only in SCC samples ($p=0.014$), but not in BCC and AK samples ($p=0.173$ and 0.314, respectively). Association analysis showed that the frequencies of Smad2 and Smad4 CNVs were correlated with the severity of skin abnormalities ($p=0.002$ for Smad2 and $p=0.029$ for Smad4).
Conclusions CNVs of Smad2 are associated with SCC and BCC, while CNVs of Smad4 are associated with SCC but not BCC.

Keywords Smad2 · Smad4 · Copy number variations · Basal cell carcinoma · Squamous cell carcinoma · Actinic keratosis

*These authors contributed equally to this work.
§These authors contributed equally to this work.

Y. Shao · J. Zhang · J. Wan · W. Zhang (✉) · B. Yu
Shenzhen Key Lab for Translational Medicine of Dermatology
Shenzhen-PKU-HKUST Medical Center
1120 Lian-Hua Road, Fu-Tian District
Shenzhen, Guangdong 518036, China
e-mail: zhangwei@alumni.ust.hk

Y. Shao · J. Zhang · J.Wan · B. Yu
Biomedical Research Institute
Shenzhen-PKU-HKUST Medical Center
Shenzhen, Guangdong, China

Y. Shao · J. Zhang · B. Yu
Department of Dermatology
Shenzhen Hospital Peking University
Shenzhen, Guangdong, China

R. Zhang
Department of General Surgery
Taizhou Municipal Hospital
Zhejiang Province, China

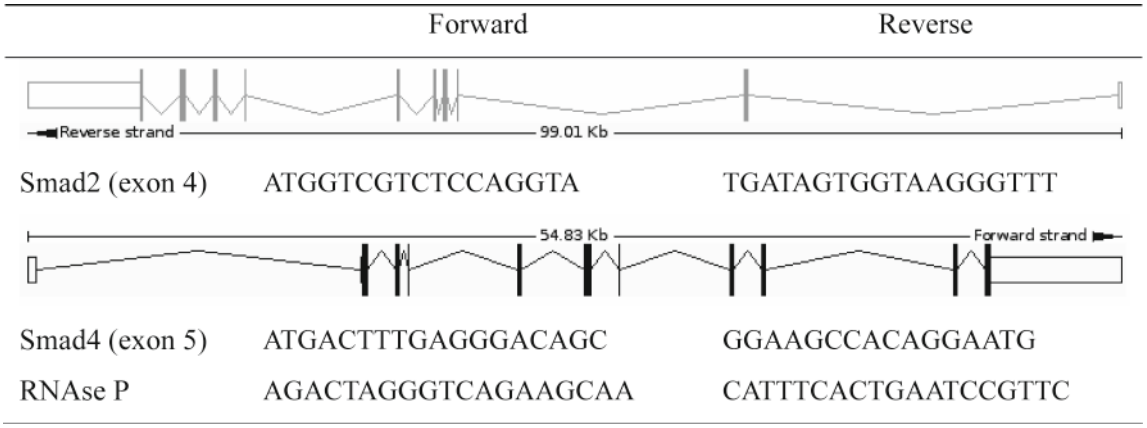
J. Wan · W. Zhang
Division of Life Science
Hong Kong University of Science and Technology
Hong Kong, China

W. Zhang
JNU-HKUST Joint Lab
Ji-Nan University
Guangdong, China

Introduction

Smad transcription factors were identified as the signalling mediators of the transforming growth factor ß (TGF ß) superfamily, which consists of three major subfamilies: TGFß, bone morphogenetic proteins (BMPs) and activins/inhibins [1]. The TGFß superfamily directly activates the Smad signalling pathway, in addition to other Smad-independent pathways. The Smad family of proteins can be divided into three functional groups: the receptor-activated

Table 1 Primers for initial quantification of copy numbers



Smads (R-Smads), common mediator Smads (Co-Smads) and the inhibitory Smads (I-Smads). The R-Smads include Smad1, Smad2, Smad3, Smad5 and Smad8. Smad6 and Smad7 are I-Smads, while Smad4 is the only mammalian Co-Smad identified thus far which mediates signals from both the TGFß/activin and BMP signalling pathways [2].

The TGFß signalling pathway plays an important role in tumour suppression, primarily via growth inhibition, apoptosis and maintenance of differentiation. Aberrations in components of the TGFß signalling pathway were observed in the majority of human epithelial cancers (>85%) including pancreatic, colon, breast, prostate and lung [3]. For skin cancer, expression of Smad2 was found to be lost in almost all human skin squamous cell carcinomas (SCCs) examined, suggesting that Smad2 plays a tumour suppressive role [4]. Likewise, deletion of Smad4 in multiple murine tissues results in spontaneous cancers including skin cancer [5, 6]. It also has been reported that copy number variations (CNVs) of SMAD2 and Smad4 were associated with cancer pathogenesis in the recent array-based study [7, 8].

CNVs were originally defined by the presence of variable numbers of copies of large, multi-kilobase genomic regions in the genomes of different individuals [9]. However, recent high-resolution genome maps have revealed smaller CNVs among healthy humans [9, 10], thus extending the definition of CNVs to the length of regions being as short as several hundred bases. Several methodologies, such as the most commonly used array-based comparative genomic hybridisation (aCGH), were utilised for genome-wide CNV detection and genotyping. CNVs have been discovered to have phenotypic consequences and have been associated with various types of cancers over the past few years [11]. However, few CNV studies were performed in skin malignancies [12, 13].

Skin cancers are divided into melanoma and non-melanoma. Non-melanoma, which is about 20 times more common than melanoma, includes basal cell carcinoma (BCC), SCC and other types of skin cancer. Actinic keratosis (AK) is considered the earliest stage in the development of skin cancer and has the potential to progress to SCC. In our study, we examined the CNVs of Smad2 and Smad4 in 195 paired samples including BCC, SCC and AK. We found that CNVs of Smad2 are associated with SCC and BCC, while CNVs of Smad4 are associated with SCC but not BCC.

Materials and methods

Controls and patient samples

Surgically resected tumour tissues and adjacent normal tissues were collected from 67 SCC, 85 BCC and 43 AK patients. The study was approved by the ethical committee of Peking University Shenzhen Hospital. The individuals gave their written informed consent. The investigations were conducted according to the Declaration of Helsinki principles.

DNA extraction and quantification of copy numbers

Genomic DNA was isolated from the tissues using the Genomic DNA Extraction Kit (Innogent, Shenzhen, China) according to the manufacturer's instruction. Quantitative PCR was performed through BioRad Chromo4 real-time PCR system. Average copy numbers of RNAse P in normal candidates (copy numbers=2) were used as the control [14]. The copy numbers of Smad2 and Smad4 were calculated by using the comparative C(T) method [15]. Cut-off values of 0.33, 0.67 and 1.33 were used to define the copy numbers as 0, 1 and 2 respectively. The primers for initial quantification are listed in Table 1. Standard curves of the primers are shown in Fig. 1 and calculation of primer efficiency is shown in Table 2. The primers for validation of Smad2 and Smad4 are listed in Table 3. Statistical analysis was performed using chi-square test or Fisher's exact test. Association analysis was performed using the linear-by-linear association test. *P*-values less than 0.05 were considered statistically significant.

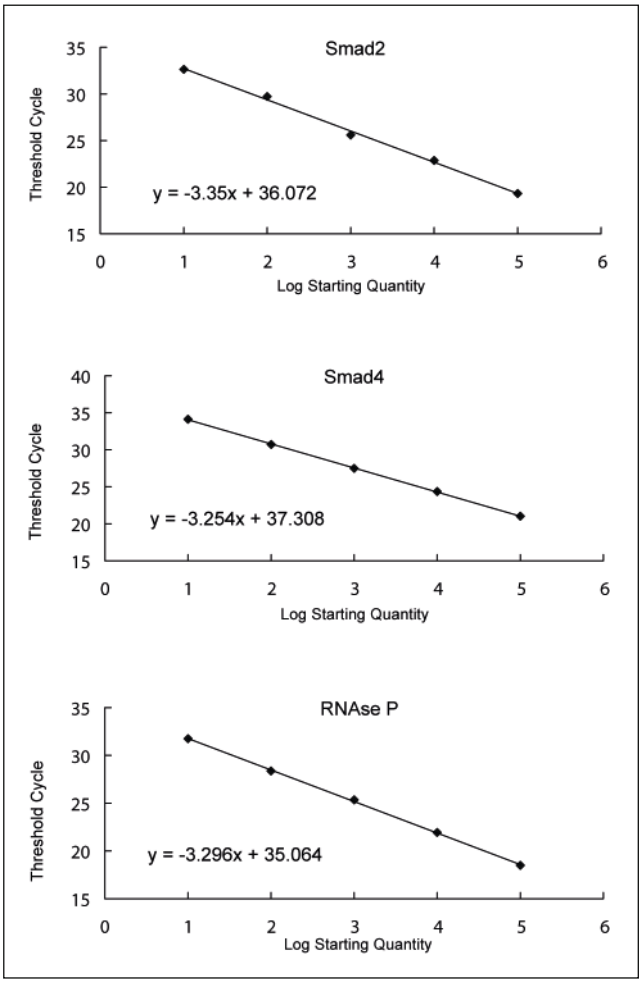


Fig. 1 Standard curves of the primers

Results

Table 4 shows CNVs of Smad2 in skin samples. A total of 195 paired samples were examined. Statistical differences were observed in SCC and BCC samples as compared with the normal tissues ($p<0.05$). However, there was no significant difference between AK samples and controls ($p=0.152$). For Smad4, statistical difference was observed only in SCC samples ($p=0.014$), but not in BCC and AK samples ($p=0.173$ and 0.314, respectively) (Table 5). The initial quantification of Smad2 and Smad4 was further validated by two additional sets of primers and similar results were obtained (Table 6).

Table 2 Calculation of primer efficiency

Genes	Dilutions	Log	Ct	Slope	Efficiency
	1	1	32.63		
	10	2	29.72		
Smad2	100	3	25.59	-3.35	0.98841698
	1000	4	22.86		
	10000	5	19.31		
	1	1	34.12		
	10	2	30.73		
Smad4	100	3	27.48	-3.254	1.02914964
	1000	4	24.37		
	10000	5	21.03		
	1	1	31.75		
	10	2	28.37		
RNAse P	100	3	25.34	-3.296	1.01093512
	1000	4	21.93		
	10000	5	18.49		

For both Smad2 and Smad4, The highest frequencies of CNVs were observed in SCC samples, while the lowest frequencies were observed in AK samples. Since SCC is known to be more malignant than BCC, while AK belongs to skin pre-malignancy, we then performed the association analysis among different types of abnormalities (Table 7). Statistical differences were observed for Smad2 ($p=0.002$) and Smad4 ($p=0.029$), indicating that the frequencies of CNVs may be correlated with the severity of skin abnormalities.

Next, we checked whether the mRNA levels of Smad2 and Smad4 were positively correlated with their copy numbers. Representative samples from the malignant tissues were divided into two groups: copy numbers=2 and copy number=1. As shown in Fig. 2, expressions of Smad2 and Smad4 in the samples with two copies of DNA were significantly higher than those with one copy of DNA, suggesting that CNVs have phenotypic consequences.

Discussion

CNVs have been clearly shown to have the potential to directly or indirectly influence a healthy individual's susceptibility to cancer, for example by varying the gene dosage of tumour suppressors or oncogenes [16, 17]. It is suggest-

Table 3 Primers for validation of copy numbers of Smad2 and Smad4

	Forward	Reverse
Smad2 (exon 1)	CAGTTCCGCCTCCAATCGC	GGGACCTTTTGTTCCTTCCTCTT
Smad2 (exon 11)	GCTACCACCTGCCACT	AGCCCAAACATAGACCTTA
Smad4 (exon 1)	GCTCAGTGGCTTCTCGACAAGTT	TCCCCTCACCCGCTCCCA
Smad4 (exon 12)	AGAGGAAGGGATGAAAC	GAAATACCACCACCAAA

Table 4 CNVs of Smad2 in skin samples

Population	No.	Genotype frequencies			*p*	Allele frequencies		*p*	OR (95% CI)
		Homo-deletion (copy number=0)	Hetero-deletion (copy number=1)	Wild-type (copy number=2)		Deletion	Non-deletion		
Total									
Cancer samples	152	5	17	130	<0.001	27	277	<0.001	29.5 (4.0–218.8)
Normal tissues	152	0	1	151		1	303		
SCC									
Cancer samples	67	4	11	52	<0.001	19	115	<0.001	22.0 (2.9–166.7)
Normal tissues	67	0	1	66		1	133		
BCC									
Cancer samples	85	1	6	78	0.026	8	162	0.004	–[a]
Normal tissues	85	0	0	85		0	170		
AK									
Abnormal tissues	43	0	2	41	0.152	2	84	0.155	–[a]
Normal tissues	43	0	0	43		0	86		

OR, odds ratios; 95% CI, 95% confidence interval

[a]OR and 95% CI cannot be calculated because the value of one weight variable was zero

Table 5 CNVs of Smad4 in skin samples

Population	No.	Genotype frequencies			*p*	Allele frequencies		*p*	OR (95% CI)
		Homo-deletion (copy number=0)	Hetero-deletion (copy number=1)	Wild-type (copy number=2)		Deletion	Non-deletion		
Total									
Cancer samples	152	1	11	140	0.022	13	291	0.001	13.5 (1.8–104.1)
Normal tissues	152	0	1	151		1	303		
SCC									
Cancer samples	67	1	7	59	0.014	9	125	0.002	–[a]
Normal tissues	67	0	0	67		0	134		
BCC									
Cancer samples	85	0	4	81	0.173	4	166	0.176	4.1 (0.5–36.8)
Normal tissues	85	0	1	84		1	169		
AK									
Abnormal tissues	43	0	1	42	0.314	1	85	0.316	–[a]
Normal tissues	43	0	0	43		0	86		

OR, odds ratios; 95% CI, 95% confidence interval

[a]OR and 95% CI cannot be calculated because the value of one weight variable was zero

Table 6 Compatibility between different sets of primers

	Smad2			Smad4		
	Initial primers	Exon 1	Exon 11	Initial primers	Exon 1	Exon 12
Exon 1	0.993	—	—	0.994	—	—
Exon 11/12	0.991	0.988	—	0.995	0.992	—

ed that the genes present in very small regions of CNVs are excellent candidates for evaluation in cancer pathogenesis. Examination of the CNVs for such genes helps to understand the functional consequences of these CNVs. Previous studies have shown that CNVs of SMAD2 and Smad4 were associated with cancer pathogenesis [7, 8]. However, in skin cancer, no such study has yet been performed.

In our study, we found that CNVs of Smad2 are associated with SCC and BCC, while CNVs of Smad4 are associated with SCC but not BCC. The significance of these findings was supported by some previous studies. It is reported that Smad2 was lost in almost all human skin SCCs examined [4]. The loss of Smad2 in human cancers suggests that Smad2 plays a tumour suppressive role. The

Table 7 Association analysis of CNVs of Smad 2 and Smad4 in SCC, BCC and AK samples

Population	No.	Genotype frequencies			*p*	Allele frequencies		*p*	OR (95% CI)
		Homo-deletion (copy number=0)	Hetero-deletion (copy number=1)	Wild-type (copy number=2)		Deletion	Non-deletion		
Smad2									
SCC	67	4	11	52		19	115		
BCC	85	1	6	78	0.002	8	162	<0.001	
AK	43	0	2	41		2	84		
Smad4									
SCC	67	1	7	59		9	125		
BCC	85	0	4	81	0.029	4	166	0.021	
AK	43	0	1	42		1	85		

OR, odds ratios; 95% CI, 95% confidence interval

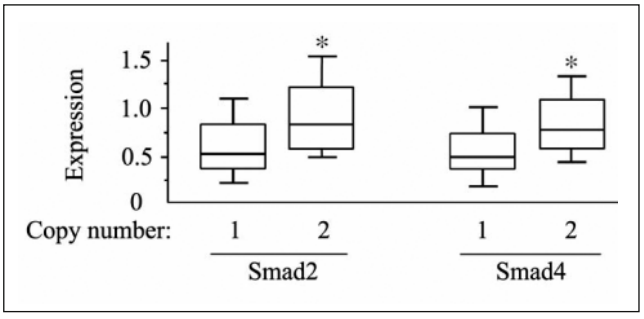


Fig. 2 mRNA levels of Smad2 and Smad4 in malignant tissues. Total RNA was isolated from skin samples, and then reverse transcribed. Quantitative PCR was used to measure the relative mRNA levels of Smad2 and Smad4. The average mRNA level in the samples with 2 copies of DNA was set as 1. Data from three independent experiments were analyzed by student t-test. The non-normal distribution data were presented as a 50% median (25% and 75% median) and range (max and min). Asterisk: $p<0.05$

role of Smad2 in skin carcinogenesis was further assessed by a mouse model. It has been shown that heterozygous germline Smad2 (Smad2 +/–) mice exhibited accelerated skin tumour formation [18]. In addition, while wild-type mice developed papillomas, well and moderately differentiated SCCs, Smad2 +/– mice developed only moderately differentiated SCCs with locally invasive and spindle-cell keratinocytes [18]. Somatic inactivation of Smad4 has been documented in multiple tumour types [3]. In the mouse model, deletion of Smad4 in multiple murine tissues results in spontaneous cancers [5, 6]. Epidermal-specific Smad4 deletion blocks the growth inhibitory effect of TGFß, and all Smad4 –/– mice developed spontaneous skin tumours including primarily SCCs, as well as sebaceous adenomas, BCCs and trichoepitheliomas [5, 6]. The results from our study further extended the understanding of Smad2 and Smad4 in human skin malignancies. However, the functional consequences of such CNVs need to be extensively investigated in the future.

Conflict of interest The authors declare that they have no conflict of interest relating to the publication of this manuscript.

Acknowledgement The study was supported by the Shenzhen Science and Technology Project (200901013). We thank the Shenzhen Biomedical Research Support Platform for technical help.

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