WNT pathway in oral cancer: Epigenetic inactivation of WNT-inhibitors

G. PANNONE[1*], P. BUFO[2*], A. SANTORO[1*], R. FRANCO[5], G. AQUINO[5], F. LONGO[5], G. BOTTI[5], R. SERPICO[7], B. CAFARELLI[4], A. ABBRUZZESE[8], M. CARAGLIA[8], S. PAPAGERAKIS[6] and L. LO MUZIO[3]

[1]Department of Surgical Sciences, Section of Anatomic Pathology and Cytopathology, University of Foggia, Foggia; [2]Department of Surgical Sciences, Section of Anatomic Pathology and Cytopathology, University of Foggia, Foggia and IRCCS CROB-Centro di Riferimento Oncologico di Basilicata, Rionero in Vulture, Potenza; [3]Department of Surgical Sciences, Section of Oral Pathology, University of Foggia, Foggia and IRCCS CROB-Centro di Riferimento Oncologico di Basilicata, Rionero in Vulture, Potenza; [4]Department of Economic Sciences, Mathematics and Statistics, University of Foggia, Foggia; [5]Istituto Nazionale per lo Studio e la Cura dei Tumori, Fondazione 'G. Pascale', Napoli, Italy; [6]Department of Otorhinolaringology, Head and Neck Surgery and Oncology, Medical School, University of Michigan Ann Arbor, Ann Arbor, MI, USA; [7]Department of Oral Pathology, Orthodontics and Oral Surgery, Second University of Napoli, Napoli; [8]Department of Biochemistry and Biophysics, Second University of Napoli, Napoli, Italy

Received February 8, 2010; Accepted March 30, 2010

DOI: 10.3892/or_00000952

Abstract. Epigenetic DNA methylations plays an important role in oral carcinogenesis. The soluble frizzled receptor protein (SFRP) family together with WIF-1 and DKK-3 encodes antagonists of the WNT pathway. Silencing of these genes leads to constitutive WNT signalling. Because aberrant expression of ß-catenin might be associated with the epigenetic inactivation of WNT inhibitors, we analyzed, in a collection of primary OSCC with matched normal oral mucosa, the methylation status of a complete panel of genes, SFRP-1, SFRP-2, SFRP-4, SFRP-5, WIF-1, DKK-3, that are involved directly and indirectly in WNT pathway, in order to demonstrate WNT-pathway activation in the absence of ß-catenin and/or APC/Axin mutations during oral carcinogenesis. Methylation-specific PCR (MSP) was performed to study inactivation of SFRP-1, SFRP-2, SFRP-4, SFRP-5, WIF-1, DKK-3 genes in 37 cases of paraffin embedded oral cancer. This study showed that the methylation is an important epigenetic alteration in oral cancer. In particular, SFRP-2, SFRP-4, SFRP-5, WIF-1, DKK-3 revealed methylation status of their promoter in OSCC, whereas SFRP-1 showed demethylation in cancer. Fisher's exact test revealed statistically significant results ($p<0.05$) for all genes. The Wald test confirmed the statistically significant association between SFRP2-4-5 gene methylation and OSCC ($p<0.05$). SFRP-1 was also characterized by a different statistically significant epigenetic behaviour, because of it was demethylated in cancer ($p<0.05$). Statistical regression test showed high levels of sensitivity, specificity and accuracy for SFRP genes, while WIF-1 and DKK-3 have reportedly high specificity, moderate accuracy but low sensitivity. This study suggests that a cause of catenin delocalization in oral cancer could be due to WNT pathway activation, by epigenetic alterations of SFRP, WIF-1 and DKK-3 genes.

Correspondence to: Dr M. Caraglia, Department of Biochemistry and Biophysics, Second University of Naples, Via S.M. Costantinopoli 16, I-80138 Naples, Italy
E-mail: michele.caraglia@unina2.it; michele.caraglia@alice.it

Dr A. Santoro, Department of Surgical Sciences, Section of Anatomic Pathology and Cytopathology, University of Foggia, Via L. Pinto 225, I-71100 Foggia, Italy
E-mail: angelasantoro1@hotmail.it

*Contributed equally

Key words: SFRP1, SFRP-2, SFRP-4, SFRP-5, WIF-1, DKK-3, WNT pathway, methylation specific PCR, oral cancer

Introduction

WNT proteins are a large family of secreted glycoproteins activating at least three signalling pathways: the canonical WNT-pathway or WNT-ß-catenin, the non-canonical WNT-pathway or planar cell polarity (PCP) and WNT-Ca^{2+} pathway (1). The canonical pathway operates by stabilizing ß-catenin, whereas the non-canonical pathway does not require ß-catenin signalling and controls cell movement during morphogenesis (2). When the WNT pathway is in its resting state, ß-catenin is phosphorylated by glycogen synthase kinase 3ß (GSK3-ß) within a protein complex that also includes casein kinase 1, adenomatous polyposis coli (APC) and Axin (3). Phosphorylated ß-catenin is immediately degraded via the ubiquitin proteasome pathway. WNT binding to Frizzled (Fz) results in the activation of Dishevelled (Dsh), which inhibits the activity of GSK3-ß, resulting in dephosphorylation and stabilization of ß-catenin, enabling it to accumulate within the nucleus, where it interacts with members of the T-cell factor/lymphocyte enhancer factor (TCF/LEF) family of trans-

cription factors to stimulate the expression of target genes (4). In sum, the canonical pathway translates a WNT signal into the transient transcription of a TCF/LEF target gene programme (5).

The main receptor of secreted WNT proteins at plasma-membrane is the above-mentioned protein Frizzled (Fz), but other proteins are required for proper WNT signalling. The Fz co-receptors are: LRP-5, LRP-6, Derailed (Drl) and the related tyrosine kinase protein (Ryk).

There are several WNT-antagonists that may be classified in two types: a) the one that interfere with WNT activity by binding to low-density lipoprotein receptor-related proteins (LRP-5 or LRP-6), including Sclerostin and Dickkopf (DKK) proteins, and b) the other one that interact directly with WNT proteins, including WIF-1, Cerberus and secreted Frizzled-related proteins (SFRPs). SFRPs comprise a family of highly conserved glycoproteins with crucial role in cell proliferation and differentiation, epithelial-mesenchymal communication and embriogenesis (6-8). SFRPs share structural similarities with the Frizzled receptor family of proteins and antagonize the WNT pathway at the level of receptor-ligand binding (9,10). Dickkopf (DKK) inhibitor binds to low-density lipoprotein receptor-related protein-6 (LRP-6), a co-receptor for WNT, and inhibits the WNT signaling pathway. DKK interacts with the co-receptors LRP-5/6 and inhibits signalling by disrupting the binding of LRP-6 to the WNT/Fz ligand-receptor complex (11,12).

WIF-1 is a secreted inhibitor of WNT signalling. WIF-1 expression results in cell growth inhibition via G1 arrest. Mechanisms of WIF-1-induced G1 arrest include (a) SKP2 (SKP2 gene contains two TCF/LEF-1 consensus binding sites within the promoter) down-regulation leading to p27/Kip-1 accumulation and (b) c-myc down-regulation releasing p21/WAF-1 transcription (13).

Although dysregulation of the WNT pathway via ß-catenin is a frequent event in several human cancers (14,15), its potential implications in oral cancer are largely unexplored. Chronic activation of WNT target gene program in human cancers may be due to different molecular events: i) mutations in APC or Axin1 genes resulting in the production of truncated scaffold proteins losing the capacity to bind ß-catenin (16); ii) mutation of the conserved serine/threonine phosphorylation sites at the amino-terminus of ß-catenin (17,18); iii) loss of WNT inhibitors through epigenetic silencing (19,20).

In oral cancer mutations of APC, Axin1 and ß-catenin genes are infrequent (16), however, delocalization of ß-catenin in precancerous oral lesions and in oral cancers have been frequently reported in current literature (21-23).

The present study investigated the methylation status of SFRP-1, SFRP-2, SFRP-4, SFRP-5, WIF-1, DKK-3 in a collection of primary OSCC with matched normal oral mucosa, in order to demonstrate WNT-pathway activation in the absence of ß-catenin and/or APC/Axin mutations during oral carcinogenesis.

Materials and methods

Study population and clinicopathological data. Thirty-seven cases of formalin-fixed, paraffin-embedded OSCC with relative controls of normal oral epithelium were retrieved from the files of National Cancer Institute of Naples, Italy; all patients underwent oral maxillo-facial surgery without previous treatment between 2005 and 2007. All patients or their relatives gave their informed written consent. Demographical and clinical data together with follow-up status were extracted from clinical records and reported in Table I. The histopathological diagnosis, reporting the grade and stage of all OSCCs, were made and carefully reviewed at the University of Foggia, Department of Surgical Science, Section of Anatomic Pathology and Cytopathology. Tumour extent determined from clinical records, computed tomography and magnetic resonance imaging data, was revised and classified according to the 2002 TNM classification. Special care was taken in assessing tumour nuclear grade on paraffin-fixed, haematoxylin and eosin (H&E)-stained sections and in defining it by appropriate grading system (20). The study patient population consisted of 29 males and 8 females, with a mean age of 67 years (range, 40-92 years), who had a complete and long-term follow-up information available. The mean follow-up time of the studied cases was 21 months. All the cases were analysed by methylation specific PCR (MSP) for WNT-pathway to establish a diagnostic epigenetic profile in oral cancer.

DNA extraction, sodium bisulfite modification and methylation specific PCR (MSP). Following careful examination of haematoxylin-eosin stained slides, we selected tissue sections with the greatest proportion of malignant tissue. Paraffin blocks with corresponding normal epithelium distant from tumour were also selected. Five 10-μM sections were cut from each formalin-fixed, paraffin-embedded tumour sample and transferred into micro centrifuge tubes.

The paraffin was dissolved using xylene followed by two washes with 100% ethanol and one wash with phosphate-buffered saline. The samples were then incubated in lysis solution [proteinase K (Qiagen), 20 mg/ml, 50 μl; 1 M Tris HCl solution, 10 μl; 0.5 M EDTA, 2 μl; 10% SDS 100 μl; distilled water 838 ml] overnight at 55˚C. De-cross linking was performed by adding NaCl (final concentration 0.7 M) and incubating at 65˚C for 4 h. DNA was recovered using the Wizard DNA clean-up kit (Promega, Madison, WI) according to the manufacturer's protocols.

Negative controls were represented by two cases of thyroid papillary cancer. To test the integrity of isolated DNA the wide housekeeping haemoglobin gene was amplified by PCR and visualized by gel electrophoresis for both control and pathological samples. The haemoglobin gene primers used were: forward, 5'-GAA GAG CCA AGG ACA GGT A-3'; and reverse, 5'-GGA AAA TAG ACC AAT AGG CAG-3'.

The DNA quantity was evaluated by a Nano Drop Spectrophotometer (Celbio). Sodium bisulfite modification of 100 μg DNA for each sample was performed using the EZ DNA methylation kit (Zymo Research, Orange, CA) following the manufacturer's protocol, with the addition of a 5-min initial incubation at 95˚C prior to addition of the denaturation reagent. The de-cross-linking steps in the extraction as well as the 95˚C incubation ensure more complete melting of the DNA and thus more complete sodium bisulfite conversion for these highly cross-linked formalin-fixed specimens. All methylation-specific PCRs were optimized to detect >5% methylated

Table I. Demographical and clinicopathological features of OSCC patients.

Age	
Range	40-92
Mean	67
Gender	
Male	29
Female	8
Sites	
Lip	1
Tongue	30
Oral floor (FOM)	1
Gingiva	1
Palate	1
Retromolar trigone	3
Total	37
TNM staging	
T1	
N0	13
N1	7
N2	0
Total	20
T2	
N0	9
N1	4
N2	1
Total	14
T3	
N0	0
N1	1
N2	1
Total	2
T4	
N0	0
N1	0
N2	1
Total	1
Total	
N0	22
N1	12
N2	3
Total	37
Stage	
St1	13
St2	9
St3	12 (7T1N1M0, 4T2N1M0, 1T3N1M0)
St4	3 (1T2N2M0, 1T3N2M0, 1T4N2M0)
Histological grade	
G1	4
G2	22
G3	11
Follow-up	
Alive	26
Died due to OSCC	7
Died due to other causes	0
Lost of follow-up	4
Tobacco consumption	
Non-smokers	5
≤20 cig/day	15
>20 cig/day	3
Unknown	14
Alcohol consumption	
No alcohol	4
≤500 ml/day	7
>500 ml/day	3
Unknown	23

substrate in each sample. Each experiment was performed in triplicate.

Methylated and unmethylated DNA were equally recovered from fixed material and only no degraded and valuable DNA samples were selected. The primers used for Nested-PCR to flank methylated/unmethylated (M/U) WNT-pathway inhibitors are reported in Table II.

Statistical analysis. The methylated/unmethylated status of the SFRP1, SFRP-2, SFRP-4, SFRP-5, WIF-1, DKK-3 was analyzed as a dichotomous variable. Data were studied by Fisher's exact test. A p-value <0.05 was considered statistically significant. Therefore, for each gene, logistic regression was performed, in order to verify the association between methylation status of gene promoter (covariate) and presence of cancer (response variable). The association between genes methylation and OSCC was valued by the Wald test. Finally, ROC curves have estimated specificity, sensitivity and accuracy of statistical regression test.

Results

This study showed that the methylation of SFRP-1, SFRP-2, SFRP-4, SFRP-5, WIF-1, DKK-3 is an important epigenetic alteration in oral cancer and that an epigenetic fingerprint exists in oral cancer.

In particular, SFRP-2, SFRP-4, SFRP-5, WIF-1, DKK-3 revealed methylation status of their promoter in OSCC, whereas SFRP-1 showed demethylation in cancer (Fig. 1). Statistical evaluation was performed in order to translate these data to clinical diagnostic setting.

We have evaluated the relative methylation frequencies, with their confidence intervals (CI) at 95%, in OSCC cases and in the matched controls (Table III). Data were studied by Fisher's exact test. The analysis revealed statistically significant results ($p<0.05$) for all genes.

Table II. Primers and PCR conditions for methylated (M) and unmethylated (U) genes of WNT-pathway.

Genes	Primers	mer	tm	Expected size (bp)
Methylated				
DKK-3_M_F	GGGGCGGGCGGCGGGGC	17	66	120
DKK-3_M_R	ACATCTCCGCTCTACGCCCG	20	58	
DKK-3_E_F	GTATTATAGGTGAGGAGTAGAGTTTAGTTT	30	56	417
DKK-3_E_R	TATCCTCCATCAATTCCTCAACCTC	26	56	
SFRP-1_M_F	GTTTTCGGAGTTAGTGTCGCGC	22	57	119
SFRP-1_M_R	ACGATCGAAAACGACGCGAACG	22	57	
SFRP-1_E_F	TGGTTTTGTTTTTTAAGGGGTGTTGAGT	28	57	429
SFRP-1_E_R	TCCTACCRCAAACTTCCAAAAACCT	25	56	
SFRP-2_M_F	TCGGAGTTTTTCGGAGTTGCGC	22	57	133
SFRP-2_M_R	GCTCTCTTCGCTAAATACGACTCG	24	57	
SFRP-2_E_F	AATTAGATTTAGAAAGTAGTGATTAGT	27	50	381
SFRP-2_E_R	AACCAAAACCCTACAACATCRTAAAC	26	53	
SFRP-4_M_F	GGGTGATGTTATCGTTTTTGTATCGAC	27	58	111
SFRP-4_M_R	CCTCCCCTAACGTAAACTCGAAACG	25	59	
SRFP4 E_F	GAGGGGGTGATGTTATYGTTTTTGTAT	27	57	304
SRFP4 E_R	CCCCAAACTCCAATCRACAACAAAAC	26	57	
SFRP-5_M_F	TGGCGTTGGGCGGGACGTTC	20	58	125
SFRP-5_M_R	AACCCGAACCTCGCCGTACG	20	58	
SFRP-5_E_F	GGGAGGTAGGGAGTTTTGGGGAGAA	25	58	277
SFRP-5_E_R	CCCAAATAAATAACAACCTACRCTAC	26	57	
WIF-1_M_F	CGTTTTATTGGGCGTATCGT	20	55	145
WIF-1_M_R	ACTAACGCGAACGAAATACGA	21	57	
WIF-1_E_F	TAGGGGTTTTTGAGTGTTT	19	50	385
WIF-1_E_R	ACCTAAATACCAAAAAACCTAC	22	50	
Unmethylated				
DKK-3_U_F	TTAGGGGTGGGTGGTGGGGT	20	65	126
DKK-3_U_R	CTACATCTCCACTCTACACCCA	22	64	
SFRP-1_U_F	GTAGTTTTTGGAGTTAGTGTTGTGT	25	60	126
SFRP-1_U_R	ACCTACAATCAAAAACAACACAAACA	26	60	
SFRP-2_U_F	GGGTTGGAGTTTTTTGGAGTTGTGT	25	64	139
SFRP-2_U_R	CCCACTCTCTTCACTAAATACAACTCA	25	64	
SFRP-4_U_F	GGGGGTGATGTTATTGTTTTTGTATTGAT	29	65	115
SFRP-4_U_R	CACCTCCCCTAACATAAACTCAAAACA	27	65	
SFRP-5_U_F	TGGTGTTGGGTGGGATGTTTG	21	61	126
SFRP-5_U_R	CAACCCAAACCTCACCATACAC	22	62	
WIF-1_U_F	GGGTGTTTTATTGGGTGTATTGT	23	59	154
WIF-1_U_R	AAAAAAACTAACACAAACAAAATACAAAC	29	59	

tm, temperature (˚C).

Table III. Analysis of the relative methylation frequencies in OSCC cases and in the controls as evaluated by MSP.

Gene	OSCC cases	95% CI OSCC cases	Controls	95% CI OSCC controls
SFRP-1	0.1622	0.0619-0.3201	0.8095	0.5809-0.9455
SFRP-2	0.4595	0.2949-0.6308	0.0476	0.0012-0.2382
SFRP-4	0.7838	0.6179-0.9017	0.3333	0.1459-0.5697
SFRP-5	0.5946	0.4210-0.7525	0.1429	0.0305-0.3634
WIF-1	0.1892	0.0796-0.3516	0.0000	0.0000-0.1611
DKK-3	0.2703	0.1379-0.4412	0.0476	0.0012-0.2382

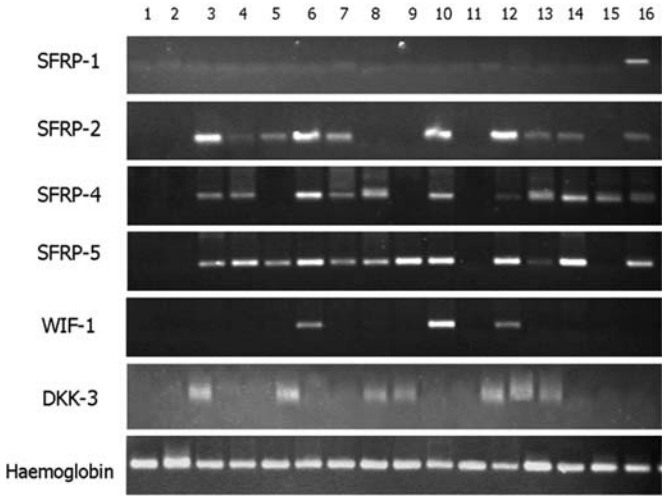

Figure 1. Representative cases of OSCCs showing high frequence of promoter methylation as evaluated by MSP. A cause of catenin delocalization could be due to WNT pathway activation by epigenetic alterations of SFRP, WIF1 and DKK3 genes in oral cancer. On electrophoretic gel, note the promoter methylation status for SFRP-2, SFRP-4, SFRP-5, Wif-1 and DKK-3 in OSCC, whereas SFRP-1 showed demethylation in cancer. For further details see Material and methods. Lanes 1 and 2, PTC, papillary thyroid cancer (negative unmethylated control). Lanes 3-16, OSCC, oral squamous cell carcinoma. Haemoglobin, house-keeping gene.

Therefore, for each gene, logistic regression was performed, in order to verify the association between methylation status of gene promoter (covariate) and presence of cancer (response variable) The results of the six estimated models are reported in Table IV.

By considering the odds ratio for all genes, the Wald test confirmed the statistical significance of the association between SFRP-2-4-5 gene methylation and OSCC ($p<0.05$; CI at 95%, Table IV). SFRP1 also showed statistically significant results, but it was characterized by a different epigenetic behaviour, because it was demethylated in cancer ($p<0.05$; CI at 95%, Table IV).

By ROC curves, we have estimated specificity, sensitivity and accuracy of statistical regression test: high levels of sensitivity, specificity and accuracy were observed for SFRP genes, while WIF-1 and DKK-3 have lower levels of sensitivity, but very high levels of specificity and a moderate accuracy. In particular, WIF-1 was totally absent of false positive cases, because of it was always unmethylated in controls.

Discussion

Oral squamous cell carcinoma (OSCC) is one of the most commonly occurring cancers of the head and neck region (24). Although it has been suggested that OSCC arises through the accumulation of multiple genetic changes with a complex multi-step process (25-27), the precise pathogenetic molecular pathway remains unclear and a well defined genetic fingerprint of oral cancer is still unknown. Recent studies indicate that epigenetic silencing of cancer-related genes via DNA methylation also plays an important role in OSCC carcinogenesis (28,29), though the exact molecular mechanisms are not fully understood.

Up to now, it has been thought that mutations in APC, Axin or CTNNB1 can cause constitutive signalling independent of

Table IV. Logistic regression model estimates.

								95% CI for OR	
	Variables	*ln(OR)*	Standard error	Wald	df	P-value	OR	Lower	Upper
Model 1	SFRP-1	-3.089	0.713	18.794	1	0.000	0.046	0.011	0.184
	Intercept	2.048	0.531	14.855	1	0.000	7.750		
Model 2	SFRP-2	2.832	1.076	6.928	1	0.008	16.985	2.061	139.980
	Intercept	0.000	0.316	0.000	1	1.000	1.000		
Model 3	SFRP-4	1.981	0.611	10.499	1	0.001	7.250	2.187	24.028
	Intercept	-0.560	0.443	1.594	1	0.207	0.571		
Model 4	SFRP-5	2.175	0.708	9.440	1	0.002	8.800	2.198	35.235
	Intercept	-0.182	0.350	0.272	1	0.602	0.833		
Model 5	WIF-1	7.846	22.843	0.118	1	0.731	2554.946	0.000	7.1E+22
	Intercept	0.357	0.285	1.572	1	0.210	1.429		
Model 6	DKK-3	2.002	1.090	3.378	1	0.066	7.407	0.876	62.668
	Intercept	0.300	0.295	1.035	1	0.309	1.350		

The Wald test confirmed the statistical significance of the association between SFRP-2-4-5 gene methylation and OSCC ($p<0.05$; CI at 95%). SFRP-1 also showed a statistically significant result, but it was characterized by a different epigenetic behaviour, because of it was demethylated in cancer ($p<0.05$; CI at 95%). Statistical regression test showed high levels of sensitivity, specificity and accuracy for the SFRP genes, while WIF-1 and DKK-3 have lower levels of sensitivity, but very high levels of specificity and a moderate accuracy. In particular, WIF-1 was totally absent of false positive cases, because it was always unmethylated in controls.

the upstream signal from WNT. Previous studies have shown that APC. CTNNB1 and Axin are frequently mutated in different types of human cancers (16), as well as colorectal cancer, gastric cancer, liver cancer and pancreatic neoplasia; however, mutations in APC or CTNNB1 are rarely found in OSCC cell lines and in OSCC (21-23).

Nevertheless, mutations of CTNNB1 (ß-catenin), the crucial center of the WNT pathway, have not been reported in oral cancer; whereas, its accumulation in the cytoplasm and nucleus is frequently observed in various cancers (30). Catenin delocalization has been reported in pre-malignant lesions of the oral cavity and in OSCC (21-23). In previous studies, we have reported that the distribution of ß-catenin is different in OSCC cells than in normal mucosal cells. Whereas normal oral epithelium shows exclusively membranous ß-catenin immunostaining (31), OSCC leaks membrane-bound ß-catenin and reveals a corresponding increase in cytoplasmic localization and a sporadic nuclear accumulation (32). Other authors also demonstrated that aberrant expression of ß-catenin is inversely correlated with differentiation and is significantly associated with invasion and poor prognosis (33,34).

Therefore, the above evidence may demonstrate that canonical WNT pathway may be activated in OSCC independently of constitutive signalling. Because aberrant expression of ß-catenin might be associated with the epigenetic inactivation of WNT inhibitors, we analyzed the methylation status of a complete panel of genes, including SFRP-1-2-4-5, WIF-1 and DKK-3, that are involved directly and indirectly in the WNT pathway.

This study, together with the results of Sogabe *et al* (35), highlights the importance of epigenetic inactivation in OSCC. Sogabe *et al* (35), studied only SFRP class of WNT-pathway inhibitors, our research focused on the two known classes of receptor and non-receptor WNT inhibitors, showing that a panel of these genes may be useful in clinical practice separating normal oral epithelia from the cancerous ones if their DNAs were analysed by methylation specific PCR technique.

There is a strong correlation between the ability of the WNT-1 gene to induce ß-catenin accumulation and its transforming potential *in vivo*, suggesting that the WNT-1 gene activates an intracellular signalling pathway that can induce the morphological transformation of cells.

As reviewed by Bovolenta *et al* (36), down-regulation of SFRP-1 and SFRP-3 has been observed in a large proportion of invasive carcinomas such as breast (37,38), gastric (39), cervical (40), hepatocellular (41) and prostate tumours (42). The down-regulation of SFRP genes in cancer is due to deletions or epigenetic silencing.

However, SFRPs have been also reported to have tumour-promoting activities, in many cases linked to the role in apoptosis as reviewed by Rubin *et al* (43).

Briefly, in our present study, we have shown that epigenetic silencing of multiple genes is a common event in OSCC. Therefore, contrary to Sogabe *et al* (35) who observed that SFRP-1 together with SFRP-2 and SFRP-5 were methylated in OSCC, we have noted an opposite epigenetic behaviour for SFRP-1, because it was significantly demethylated in cancer ($p<0.05$).

Finally, our study demonstrates that a cause of catenin delocalization could be due to WNT pathway activation, by epigenetic alterations of SFRP, WIF-1 and DKK-3 genes. Our results not only shed light on a molecular mechanism contributing to OSCC tumourigenesis, but also suggest that employing of an epigenetic fingerprint, together with the classical histopathological parameters, may improve the current diagnostic tools, but also contribute indirectly to therapeutics as predictor of choice for the correct clinical management of oral neoplastic and preneoplastic lesions.

Acknowledgements

First of all, we thank all our patients or their relatives for their voluntary participation in this study. Then, a particular thanks to Maria Carmela Pedicillo and Simona Cagiano, for their fundamental technical contribution. In addition, we thank Oscar Fernando D'Urso, Biotengen, Lecce, for precious collaboration in MSP technique. The main source of funding for conducting this research investigation comes from the Department of Surgical Pathology, University of Foggia, Foggia, Italy and the San Antonio Cancer Institute, University of Texas Health Science Center at San Antonio, TX, USA (grants #122336 and 122897, S.P.). Finally, this work was partly supported by Fondazione Caripuglia, Bari, Italy and by MURST-PRIN (Italian Ministry of University Science and Technology, protocol n. 2005069443-03 and n. 2005062791-004).

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