

# Significance of P16<sup>INK4A</sup> hypermethylation gene in primary head/neck and colorectal tumors: it is a specific tissue event? Results of a 3-year GOIM (Gruppo Oncologico dell'Italia Meridionale) prospective study

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**Background:** Methylation of the p16 promoter is one of the most frequent mechanisms of gene inactivation; its incidence is extremely variable according to the type of tumor involved. Our purpose was to analyze the hypermethylation of the p16 promoter in laryngeal squamous cell carcinomas (LSCC), salivary gland (SG) tumors and in colorectal cancer (CRC), to detect any possible association with the clinicopathological features and to determine the prognostic significance of the p16 gene in the tumors analyzed.

**Patients and methods:** The hypermethylation of the p16 promoter was prospectively analyzed, by MSP, in a consecutive series of 64 locally advanced LSCC patients, in a consecutive series of 33 SG tumor patients and in a consecutive series of 66 sporadic CRC patients.

**Results:** Hypermethylation was observed in 9% of the LSCC cases, in all cases of SG cancer and in 21% of the CRC cases. No significant association was observed between p16 hypermethylation and clinicopathological variables in all the tissue samples analyzed. Moreover at univariate analysis p16 mutations were not independently related at disease relapse and death in LSCC and CRC.

**Conclusions:** The results of this study suggest that the lack of p16 function could happen in advanced stage of SG tumors.

**Key words:** P16 hypermethylation, laryngeal squamous cell carcinoma, salivary gland cancer, colorectal cancer

## introduction

DNA methylation is an epigenetic phenomenon that affects both normal DNA function and interaction with proteins [1]. In normal conditions, methylation is a control mechanism for the tissue expression of specific genes and their contemporaneous silencing in the cells of different tissues [2]. This perfect epigenetic balance of normal cells is altered when the cells become tumoral [2]. Aberrations in DNA methylation patterns are, in fact, now recognized as a hallmark of human cancer. DNA methylation of promoter-associated CpG islands is an alternate mechanism to mutation in silencing gene function,

and affects tumor-suppressor genes such as p16 and RB1, growth and differentiation controlling genes such as ER, and many other genes involved in the apoptotic pathways, in DNA repair, in the cell cycle, in cell adhesion, and so on [3]. One of the most common alterations, occurring early in tumorigenesis, is the hypermethylation of the oncosuppressor gene promoters [2]. A great many studies have reported that during the carcinogenetic process, hypermethylation often occurs in order to silence one or both alleles [4, 5]. Methylation of the p16 promoter is one of the most frequent mechanisms of gene inactivation; its incidence is extremely variable according to the type of tumor involved. P16 inhibits the cyclin D1-Cdk 4/6 complex, which is responsible for the phosphorylation of the Rb protein, thus bringing about a block in the cell cycle in phase G1 [6].

In laryngeal squamous cell carcinomas (LSCC) p16 alterations (deletion, mutation or down-regulation caused by

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the promoter hypermethylation) appear to be one the mechanisms implicated in neoplastic transformation and progression [7]. It has been reported that in 22% of cases, p16 is inactivated by mutations or deletions in homozygosis and in 7% by promoter hypermethylation [7]. Nevertheless, the effect of p16 promoter methylation in the development of salivary gland (SG) carcinomas remains unclear. Furthermore, little is known about the promoter methylation role in adenoid cystic carcinomas (ACC) of the salivary gland.

It has been suggested that altered expressions of pRb and p16 are involved in colorectal cancer, indicating that these two genes, either alone or combined, might be useful as markers of an unfavorable outcome [8]. Cui et al. [9] have observed expression loss of the gene p16 in 62% of their cases of CRC. Moreover, several analyses of the silencing of this gene by promoter methylation have reported different results [10–12].

The aim of this study, therefore, was to analyze the hypermethylation of the p16 promoter in head and neck tumors (locally-advanced LSCC and SG tumors) and in gastrointestinal tumors (CRC), to detect any possible association with the clinicopathological features of patients with these diseases and finally to determine the prognostic significance of the p16 gene in the various tumors analyzed.

## patients and methods

### study design

LSCC patients. A prospective study was performed on paired tumor and normal laryngeal tissue samples from a consecutive series of 64 patients with stage III (32 T3 N0, 4 T2 N1 and 6 T3 N1) and stage IV (8 T4 N0, 3 T4 N1, 8 N2 and 3 N3), undergoing potentially radical surgical resection for primary tumor of larynx between April 1990 and December 1998. Briefly, the following inclusion criteria were used: (a) electively resected primary LSCC; (b) processing of fresh paired normal mucosa-tumor samples within 30 min after tumor removal; and (c) available DNA from normal and tumor tissue. In order to avoid evaluator variability in the patients, all resection specimens and microscopic slides were meticulously examined by two independent pathologists (RMT and VM) who were not aware of the original diagnosis or of the results of the molecular analysis. The complete excision of the primary tumor was histologically proven by examination of the resected margins. All tumors were histologically confirmed to be squamous cell carcinomas. In addition, the pathologists assessed tumor site (supraglottis, glottis, subglottis), tumor size, pathological stage, tumor grade (histological differentiation), and the presence or absence of lymph node metastases. Tumors were staged according to the American Joint Committee on Cancer and graded as well (grade 1), moderately (grade 2) and poorly (grade 3) differentiated. Lymph node involvement was evaluated clinically and histologically without considering the number or location of nodes. Written informed consent was obtained from all patients included in this study. A standard questionnaire of more than 50 clinicopathologic and research variables was available for each patient at the moment of surgery and was maintained on a computerized data base. Postoperatively, all patients were checked at 3-monthly intervals for the first 2 years, at 6-monthly intervals for the next 2 years, and annually thereafter. The follow-up program included a clinical examination, blood tests (including CEA and SCC assays) and annual chest X-ray. Disease relapse (local recurrence or distant metastases) was confirmed histologically where possible.

SG cancer patients. A prospective study was performed on tumor samples from a consecutive series of 33 patients who underwent resective surgery for primary operable SG tumor at the Department of Oncology,

University of Palermo, Italy. All resection specimens and microscopic slides were examined by two independent pathologists (RMT and VM) who were blinded to the original diagnosis and the results of molecular analyses. The complete excision of the primary tumor was histologically proven by examination of the resected margins. The study material included 28 PAs, four cystic adenocarcinomas and one carcinoma ex-pleomorphic adenoma. Tissues were fixed in 70% ethyl alcohol and paraffin-embedded. At least three single sections were analyzed from each paraffin-embedded tissue. One of the sections was used for microdissection followed by mutational analysis, while the others were used for immunohistochemistry experiments.

CRC patients. A prospective study was performed on paired tumor and normal colon tissue samples from a consecutive series of 66 previously untreated patients, undergoing resective surgery for primary operable sporadic CRC at a single institution (Department of Oncology, University of Palermo) from January 1988 to December 1992.

Briefly, none of the patients had any history of previous neoplasias, synchronous or metachronous CRC. All tumors were histologically confirmed to be colorectal adenocarcinomas. Patients with Dukes' stage A and B sporadic CRC were treated with surgery alone, whereas all the 28 patients with Dukes' stage C received adjuvant chemotherapy with 5-FU, leucovorin and levamisole since in the period before 1991 hardly any of the patients had received adjuvant treatment. Patients with non-radical surgery and/or distant metastases were treated with 5-FU and leucovorin. Postoperatively, all patients were checked at 3-monthly intervals for the first 2 years, at 6-monthly intervals for the next 2 years, and annually thereafter. Written informed consent was obtained from all patients included in the study.

### tissue handling and DNA extraction

LSCC. Multiple samples (four to eight) of the primary tumor tissue were taken from at least four different tumor areas (including the core and the invasive edge of the tumor). The portion of primary tumor was obtained by superficial biopsy of either the tumor bulk or the edge of the malignant ulcer for more infiltrative cancer. All tissues were carefully trimmed to remove as much non-neoplastic tissue as possible, avoiding the non-viable areas. Furthermore, samples of normal mucosa (as confirmed by histology) were taken from a macroscopically uninvolved area 2–5 cm away from the tumor site, to be used as control for biomolecular and flow cytometric analysis. The tissues were bisected, one half of each sample was processed for pathological examination, and the remaining half of the sample pool was immediately frozen and stored at  $-80^{\circ}\text{C}$  until analyzed. Where present, areas with a high content of non-neoplastic cells were removed from the frozen block with a scalpel. Genomic DNA was extracted using the QIAamp Tissue Kit (Qiagen, Hilden, Germany) with the standard protocol from primary LSCC and normal laryngeal specimens.

SG cancer. Sections, 5- $\mu\text{m}$ , of paraffin-embedded tissue specimens, prepared using a microtome, were mounted on the supporting Laser Pressure Catapulting (LPC) membrane placed on the slide. The samples were pretreated with xylene for 10 min and rehydrated using decreasing grade alcohols (100%, 95% 50% ethanol) and  $\text{H}_2\text{O}$ . The slides were then stained with hematoxylin and eosin and dehydrated using increasing graded alcohol. LPC was performed using a Zeiss inverted microscope PALM Laser Micro-Beam System UV laser at 337 nm. Before performing microdissection, 1  $\mu\text{l}$  of mineral oil was placed on the samples. The areas to be dissected were selected by means of extremely high-precision microcuts (the specimens ranged from as little as 1  $\mu\text{m}$  to 1000  $\mu\text{m}$  in diameter). LPC dissection was performed using a few shots each of 100  $\mu\text{m}$  in diameter. After catapulting, the material was removed from the cap for genetic analysis. Genomic DNA from tumor and normal specimens was extracted using the QIAamp Tissue Kit (Qiagen, Hilden, Germany) following standard protocol.

CRC. Multiple samples of the primary tumor tissue were taken from different tumor areas (including the core and the invasive edge of the tumor). Furthermore, multiple samples of normal mucosa (as confirmed by histology) were taken from macroscopically uninvolved areas 20–40 cm away from the tumor site, to be used as a control for biomolecular and flow-cytometric analysis. The tissues were bisected; one half of each sample was processed for pathological examination and the remaining half of the sample pool was immediately frozen and stored at  $-80^{\circ}\text{C}$  until analyzed. The adequacy of the material was checked on frozen tissue sections and only tissue samples with more than 80% tumor content were utilized in subsequent biomolecular analysis. Evaluation of each biomolecular was performed independently by researchers who had no knowledge of the clinical data regarding the samples. Genomic DNA was extracted using the QIAamp Tissue Kit (Qiagen, Hilden, Germany) with the standard protocol from primary CRC and normal colorectal specimens.

p16 methylation assay. One mg of genomic DNA was treated with sodium bisulfite at  $50^{\circ}\text{C}$  for 18 h using CpG genome modification kit (ONCOR, Gaithersburg, MD). The modified DNA was amplified by PCR using specific primers (Table 1) to distinguish methylated and unmethylated regions (MD). The amplification products were verified by 2% agarose gel electrophoresis and ethidium bromide staining.

### statistical analysis

Fisher's exact test (StatXact Turbo, Cytel Software Corporation, Cambridge, MA, USA) was used to evaluate the associations between biological variables. The relationship of different prognostic variables with disease-free survival (DFS) and overall survival (OS) was assessed univariately by means of the Kaplan–Meier method. Survival time was calculated from the date of surgery to the date of death (cancer-related causes) or last follow-up, with times censored for patients dying of causes unrelated to the specific tumor. DFS was measured from the day of primary surgery to the date of the first relapse (loco-regional or metastatic). Significant differences among survival curves were checked by the log-rank test and Wilcoxon test, or a trend test where appropriate. *P* values less than 0.05 were considered significant.

## results

### clinicopathologic variables

The LSCC study group was made up of 63 men and one woman and the mean age was  $64.5 \pm 8.3$  years (range 42–85 years). The histopathological features of these patients are listed in Table 2.

The CRC series of patients comprised 32 females and 34 males with a median age of  $69 \pm 11.9$  years (range 35–87). The histopathological features of these patients are listed in Table 3.

### methylation status of the p16 gene

For LSCC patients, hypermethylation of the p16 gene promoter was assessed in 9% (six of 64) of analyzed tumors. For SG cancer patients, the analysis of the methylation status of the p16<sup>INK4a</sup> gene promoter revealed that 14% (four of 28) of the PAs examined showed aberrant methylation within the CpG island. Furthermore, in all five cases of carcinoma examined, we found

hypermethylation of the p16<sup>INK4a</sup> promoter. For CRC patients, 14 of the 66 colorectal cancer patients (21%) showed a hypermethylation status of the p16<sup>INK4a</sup> gene promoter.

No significant association was observed between p16 hypermethylation and clinicopathological variables in all the tissue samples analyzed.

No significant difference were observed in relapse or death for patients with p16 hypermethylation gene in LSCC and CRC.

## discussion

The p16<sup>INK4a</sup> (CDKN2A/MTS1) putative tumor suppressor gene encodes a cyclin dependent kinase inhibitor, which plays an important role in the regulation of the G1/S phase cell cycle checkpoint. A high frequency of various p16 gene alterations was consequently observed in many primary tumors. P16 can be inactivated by different mechanisms: homozygous deletion; methylation of the promoter region; or point mutation [13].

Defects in the system controlling the cell cycle can lead to an increased proliferation of cancer cells.

### head and neck cancer

Hypermethylation is a fundamental mechanism for the epigenetic inactivation of key genes in the development of head and neck tumors [14]. It has, in fact, been observed in between 7% and 47% of HNSCCs [15–17].

### LSCC

In order to investigate the molecular mechanisms responsible for the inactivation of one of the two alleles of the p16 gene in LSCC, we evaluated the methylational status of its promoter. In accordance with a previous study, we found methylation of 9%; in fact, Jares et al. in a series of 46 LSCCs, observed a p16 hypermethylation rate of 7% [18]. This suggests that although hypermethylation of the promoter region of the p16 gene has been shown to be one of the major mechanisms for the inactivation of this tumor suppressor gene [19], it may not play such an important role in LSCC. This is in line with the finding that hypermethylation of p16 is a selective phenomenon depending on the tissue of origin [20, 21]. Several studies have suggested that p16 might not be the only inactivation target in this chromosomal region and that several other oncosuppressors might also be involved [22]. In our own study we did not find any association between the p16 promoter hypermethylation and the clinicopathological features of the patients examined. Moreover in our study no significant differences were observed in relapse or death for patients with p16 hypermethylation gene. Until now, according to our results, no literature data have shown a prognostic significance of p16 hypermethylation gene in LSCC.

**Table 1.** PCR primers used for MSP analysis of p16 gene

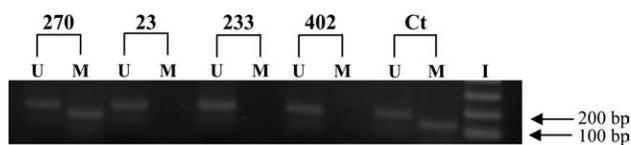
	Primers: sense 5' → 3'	Primers: antisense 5' → 3'	Anneal, °C
P16M	tta tta gag ggt ggg gcg gat cgc	gac ccc gaa ccg cga ccg taa-	61
P16U	tta tta gag ggt ggg gtg gat tgt	cca ccc caa acc aca acc ata a	66

**Table 2.** Histopathology characteristic of LSCC patients

Tumor site	
Supraglottis	30
Glottis	30
Subglottis	4
Histological grade	
G1	20
G2	31
G3	13
Size	
T2	7
T3	39
T4	18
Stage	
III	42
IV	22
Node status	
Node negative	40
Node positive	24

**Table 3.** Histopathological characteristics of CRC tumors

Tumor site	
Proximal	8
Distal	8
Rectum	50
Histological grade	
G1	5
G2	49
G3	12
Size	
≤5 cm	41
>5 cm	25
Dukes' stage	
A	16
B	19
C	30
D	1
Node status	
Node negative	52
Node positive	14



**Figure 2.** P16INK4a analysis using MSP in 4 LSCC patients. The presence of an amplified band indicates that the site is unmethylated (U) or methylated (M); ct: unmethylated (U) or methylated (M) Control; I: 100 bp ladder.

**SG cancer**

Since hypermethylation is clearly extremely important in the development of head and neck tumors, it has been decided to analyze the p16 promoter methylation status on a group of 28 pleomorphic adenomas and five salivary gland tumors. Hypermethylation of the p16 promoter was observed in 14% of

the pleomorphic adenomas and in all five cases of salivary gland tumors.

A recent study of 42 cases of PA and SG cancer has shown that p16<sup>INK4a</sup> does not present microdeletions or specific mutations in any exons, but in 28% of cases there was methylation of the p16<sup>INK4a</sup> promoter, which correlated with loss of mRNA transcription [23]. Data reported in literature regarding p16 methylation are discordant, which might depend on the different feeding habits of the patients involved in the various studies [24].

The results reported in literature are extremely varied, including those regarding the incidence of p16<sup>INK4a</sup> promoter methylation in SG tumors, which range from 11% to 47%. [25, 26]. The high percentage of our own results might depend on the fact that we used the microdissection laser technique, which made it possible to select only tumoral cells. Furthermore, reported data often regard studies conducted on Oriental populations, where this type of neoplasia is more common and the results obtained in these cases might therefore also be affected by different feeding habits.

**colorectal cancer**

Methylation of the CpG islands associated with transcriptional silencing, seems to be common in tumors of the colon [27]. In particular, a specific CpG methylator phenotype (CIMP) has been proposed that seems to be responsible for colorectal cancer progression [28]. This model is explained by the identification in the genome of sites that are preferentially methylated in tumors (MINT loci). In addition to this, CRC with CIMP have been shown to have methylation of know tumor suppressor gene such as p16 [29]. In particular, a literature review has shown a frequency of p16 hypermethylation in sporadic CRC between 20% and 50% [30–33]. In the present study, a frequency of p16 hypermethylation in 14 of 66 (21%) CRC patients has been identified. Finally, much evidence has shown that tumors with CIMP phenotype are associated with specific clinicopathological characteristics: a poor differentiation, a higher stage and a tumor in proximal colon. Unfortunately, in this study, we did not identify an association between the epigenetic alteration in p16 gene and the clinicopathological characteristics of CRC patients. The literature data of the prognostic significance of p16 hypermethylation are controversial. In fact, while San-Casla et al. [34] did not find any prognostic role of p16 hypermethylation in CRC progression, both in term of DFS and OS, other authors show that p16 hypermethylation predicts shorter survival [35, 36]. All of these discordant data might be due to several factors, such as tumor storage method (fresh/frozen tissue and paraffin-embedded blocks), the different techniques used for assessing the methylation status of p16 gene (MSP, Southern blott, RFLP, etc.), tumoral heterogeneity, or the specific features of the patient cohorts included in the study, such as histopathologic staging, grading, histological type of tumor.

In conclusion, the results of this study do not support the hypothesis that the p16 hypermethylation gene could have a predominant role in tumorigenesis pathway. Even so, we have identified a major frequency of p16 methylation in all the salivary gland tumors suggesting that the lack of p16 function

could happen in advanced stage of these specific tumors. Additional studies and larger samples are needed before our conclusions are validated.

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