# IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF MOLECULAR MARKERS INVOLVED IN HEAD AND NECK TUMOR FORMATION BY THE ANALYSIS OF THE PATTERN OF DIFFERENTIAL METHYLATION

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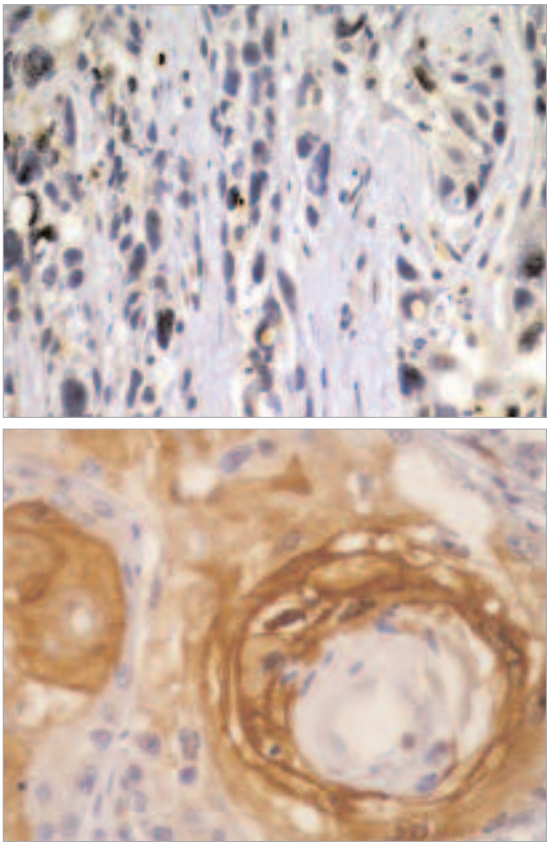
IMMUNOHISTOCHEMISTRY OF CRABP2

Squamous Cell Carcinoma of Pharynx



*Above, not detected in poorly differentiated tumor. And below, detected in well differentiated tumor*

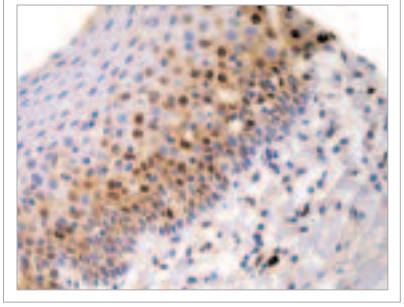
DNA methylation of the cytosine of CpG dinucleotides frequently leads to transcriptional silencing and is involved in several normal physiological conditions. However, aberrant DNA methylation is highly implicated in cancer biology, repressing the expression of genes associated with tumor formation and progression. Therefore, the analysis of the methylation pattern of CpG islands in tumors can reveal molecular markers useful for diagnosis and/or prognosis of neoplasia and lead to the identification of new therapeutic targets. The main goal of this project is to identify genes silenced in head and neck tumors by DNA methylation in their regulatory regions, which can be used as molecular markers for patients with this type of tumor. The specific aims are: (a) identify methylated genes in head and neck carcinoma cell lines, after reactivation of gene expression with a demethylation drug, by using RaSH – Rapid Subtraction Hybridization; (b) validate by micro array the gene expression reactivation by the demethylating drug in head and neck carcinoma cell lines; (c) confirm the gene expression reactivation of the selected genes by qRT-PCR in the cell lines; (d) analyze the methylation pattern of CpG islands of the selected genes in the cell lines, by DNA conversion with sodium bisulfite followed by DNA sequencing; (e) investigate the differences in the methylation patterns of the selected genes between head and neck carcinomas and normal tissues, using MSP – Methylation Specific PCR; (f) analyze the expression of the selected methylated genes in head and neck carcinomas by immunohistochemistry and correlate the results with clinical data of patients and (g) functionally characterize the genes methylated in head and neck tumors by using the yeast *Saccharomyces cerevisiae* as a model organism (for genes with homologs in yeast) or the yeast two-hybrid system (for genes without homology). In parallel, investigate the methylation pattern of LHX6 and ADAM23 in this type of tumor.

## SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

The combination of RaSH and microarray resulted in the identification of 78 genes reactivated by treatment with the demethylating agent 5-aza-2'-deoxycytidine (5-azadC) in head and neck carcinoma cell lines from different anatomic sites (pharynx, mouth floor, supraglottis and tonsils). The genes containing bonafide CpG islands in their regulatory regions and which were reactivated in at least 2 out of the 4 carcinoma cell lines used were selected for further validation by qRT-PCR. Out of the 36 genes analyzed, reactivation of gene expression was confirmed for 5 genes (*Crabp*2, *Mx*1, *Slc*15*A*3, *Capza*1 and *Ktn*1). So far, bisulfite DNA conversion assay followed by nucleotide sequencing has confirmed differential DNA methylation of the genes *Crabp*2, *Mx*1 and *Slc*15*A*3 in at least one of the head and neck carcinoma cell lines. Concerning *Crabp*2, MSP assays performed with a panel of 120 samples of head and neck carcinomas demonstrated a high frequency (65%) of DNA methylation in this gene. Protein expression of CRABP2 was then analyzed by immunohistochemistry using a Tissue Micro array of 73 tumor samples. Accordingly, it was shown in most of the cases (62%), that the head and neck carcinoma samples are negative (11%) or weakly positive (51%) for the CRABP2 protein, confirming the data obtained by MSP. Next, the expression pattern of the CRABP2 protein was compared with the global survival rates of patients. This analysis showed a statistically significant correlation between lack of the CRABP2 protein and lower survival rates of patients. Therefore, the data obtained so far have revealed that the presence of methylation in the regulatory region of *Crabp*2 that results in its silencing is a marker of poor prognosis for patients with head and neck carcinoma tumors.

IMMUNOHISTOCHEMISTRY OF CRABP2

Normal Epithelial Tissue



*Detected only in the basal layer*

In the parallel study, with the genes *Lhx*6 e *Adam*23, interesting results were obtained. For *Lhx*6, it was demonstrated that the methylation pattern of the *Dime*-6 fragment is correlated with gene silencing of the shorter isoform of *Lhx*6, suggesting that hypermethylation of *Dime*-6 is a good tumor marker for head and neck cancer. Considering *Adam*23, a correlation was found between DNA methylation and the stage of the tumors analyzed.

## MAIN PUBLICATIONS

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