

EUROPEAN SCHOOL OF MOLECULAR MEDICINE
SEDE DI NAPOLI
UNIVERSITA' DEGLI STUDI DI NAPOLI "FEDERICO II"
Ph.D. in Molecular Medicine – Ciclo III/XXI
Molecular Oncology



“Biological function of Cl 2 gene and it’s role in thyroid cancer”

Tutor:
Prof. Alfredo Fusco

Internal Supervisor:
Prof. Tomaso Russo

External Supervisor:
Prof. Laura Corbo

Coordinator:
Prof. Francesco Salvatore

Ph.D. student:
Dr. Angelo Ferraro

Academic Year: 2008-2009

Index

1 Abstract.....	2
2 Introduction.....	3
2.1 Thyroid gland.....	3
2.2 Thyroid cancer.....	4
2.3 Genetic lesions in thyroid carcinomas.....	7
2.3.1 Proto-oncogenes and oncogenes.....	7
2.3.2 Tumor suppressor genes.....	9
2.4 Cl 2 gene.....	11
3 Materials and Methods.....	18
4 Results.....	29
4.1 Cl 2 expression is drastically reduced in human thyroid carcinomas.....	29
4.2 Analysis of loss of heterozygosity (LOH) at the Cl 2 locus (3q13.2) in thyroid neoplastic samples.....	32
4.3 Methylation analysis of Cl 2 gene.....	34
4.4 Immunohistochemical analysis of Cl 2 gene expression.....	36
4.5 Cl 2 expression is also downregulated in ovary, colorectal and breast carcinomas.....	38
4.6 Restoration of Cl 2 gene expression reverts the malignant phenotype of thyroid carcinoma cell lines.....	39
4.7 The restoration of the Cl2 expression Cl2 gene inhibits the growth of thyroid carcinoma cell line KAT-4 in semisolid medium.....	41
4.8 Cl 2 affects the cell motility in vitro.....	42
4.9 Restoration of Cl 2 expression causes cell death.....	45
4.10 Generation and characterization of Cl 2 null mice.....	47
5 Discussion.....	50
6 Acknowledgements.....	54
7 Bibliography	55

To my wife

1

ABSTRACT

Here I report data to support a tumor suppressor role of the *Cl 2* gene. Indeed, I detected a drastic reduction, of *Cl 2* gene expression in almost all thyroid carcinomas analyzed, with respect to normal thyroid, with the lowest expression levels observed in the follicular variants of papillary carcinomas. Loss of heterozygosity and CpG hypermethylation at the second exon likely accounts for *Cl 2* downregulation in thyroid carcinomas. A drastic reduction in *Cl 2* expression, with respect to normal counterpart tissues, was also observed in breast, colon and ovarian carcinomas. The restoration of *Cl 2* expression in two human thyroid anaplastic carcinoma cell lines leads to the suppression of the malignant phenotype associated with a higher susceptibility to apoptosis.

The development of thyroid adenomas and ovarian carcinomas in *Cl 1* knock-out mice validates the tumor suppressor role of *Cl 2* gene. Finally, transgenic mice for RET/PTC1 oncogene crossed with the *Cl 2* knock-out mice developed much more aggressive thyroid carcinomas compared with those observed in the single mutant RET/PTC 1 mice.

Therefore, these results taken together indicate *Cl 2* as a putative tumor suppressor gene in the process of human thyroid carcinogenesis.

2 INTRODUCTION

2.1

Thyroid gland

The thyroid gland, which is one of the largest endocrine organs in humans, regulates systemic metabolism through thyroid hormones. It is composed of spherical follicles that selectively absorb iodine from the blood for the production of thyroid hormones (T3 and T4). The follicles are surrounded by a single layer of thyroid epithelial cells named follicular cells, which synthesize and secrete T3 (L-triiodothyronine) and T4 (L-thyroxine) hormones under the control of the hypothalamic–pituitary axis with negative feedback by the thyroid hormones (Kondo T, 2006) (Figure 1).

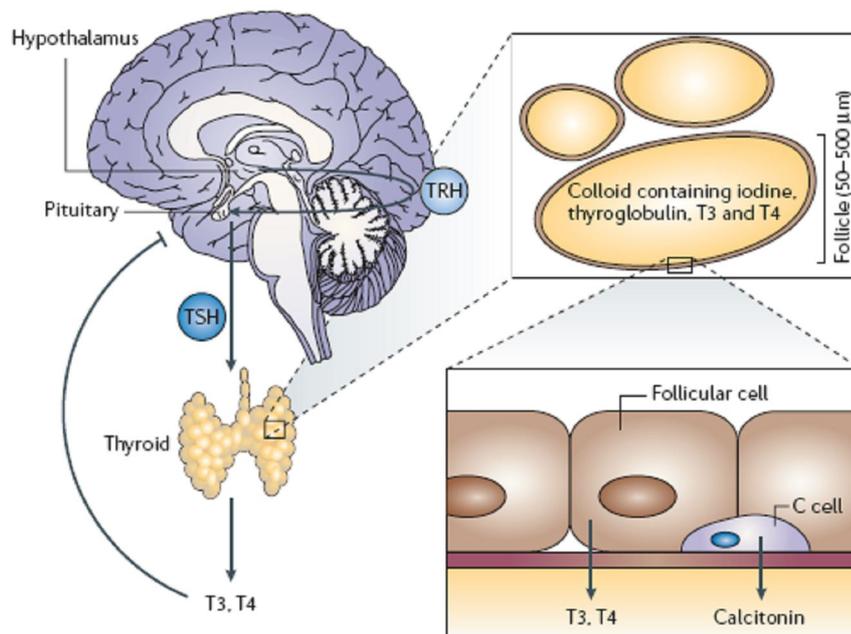


Figure 1. Schematic representation of thyroid gland structure and function. Thyroid follicular cells synthesize, store and secrete thyroid hormones T3 and T4 under the control of the hypothalamic-pituitary axis with negative feedback by the thyroid hormones.

Thyrotropin-releasing hormone (TRH), which is secreted from the hypothalamus, stimulates the release of thyroid-stimulating hormone (TSH) from the anterior pituitary gland. TSH stimulates the follicular cells to synthesize and secrete thyroid hormones. Parafollicular cells (or C cells) are in the interstitial spaces outside the thyroid follicles and produce the calcium-regulating hormone calcitonin.

2.2

Thyroid cancer

Tumors are the result of the accumulation of different modifications in critical genes involved in the control of cell proliferation. In a large number of carcinomas with worst prognosis, lesions are not diagnosed until the disease is at an advanced stage. Although various therapeutic approaches are followed in clinical practice, most of them are not life-saving. Thyroid nodules can be hyperplastic benign adenomas or malignant lesions, and can be derived from thyroid follicular epithelial cells or C cells. However, more than 95% of thyroid carcinomas are derived from follicular cells (Kondo T, 2006) and are the most common endocrine malignancies, with an estimated 25,000 new cases diagnosed annually in the United States. Conversely, only 3% of thyroid tumors, referred to as medullary thyroid carcinoma (MTC), are of C cell origin. Thyroid neoplasms represent a good model for studying the events involved in epithelial cell multi

step carcinogenesis because they comprise a broad spectrum of lesions with different degrees of malignancy diagnosed on the basis of histological and clinical parameters. Follicular cell-derived thyroid tumors include: 1) benign adenomas, which are not invasive and very well differentiated; 2) carcinomas, which are divided into well differentiated, poorly differentiated and undifferentiated types. Well differentiated thyroid carcinomas are papillary (PTC) and follicular (FTC) types, being differentiated and having a good prognosis (Kondo T, 2006; Saltman B, 2006). Most well-differentiated thyroid carcinomas are 2–4 times more frequent in females than in males and manifest in patients who are between 20-50 years of age. The papillary carcinoma is defined as a malignant epithelial tumor, showing papillary and follicular architecture and typical nuclear alterations (Hedinger C, 1989). Particularly, “occult” papillary carcinomas are slow-growing and clinically silent carcinomas. PTCs represent more than 70% of thyroid malignant tumors and occur two to three-fold more frequently in females (Salabe GB, 1994). Exposure to ionizing radiation and radioactive contamination of the environment increase the risk of developing this kind of cancer as evidenced by the effects of the atomic bombs of Hiroshima and Nagasaki (1945), nuclear testing on the Marshall Islands (1954) and Nevada (1951–1962), and the more recent nuclear accident in Chernobyl (1986) (Nikiforov Y, 1994; Kazakov VS, 1992). After the Chernobyl disaster, the effects of radiation exposure were most pronounced in children. External beam-

radiation exposure in childhood for the treatment of benign conditions of the head and neck also increased the risk of papillary carcinoma (Ron E, 1995). Differently from papillary carcinomas, which can invade local lymphnodes, follicular carcinomas are characterized by haematogenous spread and distant metastasis. FTCs represent 10% of thyroid carcinomas, are more common in females than in males and rarely associated with radiation exposure. A common subtype of papillary thyroid carcinoma is the follicular variant of papillary thyroid carcinoma (FVPTC). Microscopically, papillary carcinomas are characterized by the presence of papillae whereas the follicular variant contains a variable area of both papillary and follicular histology. (Hay ID. 1990). These tumors contain a predominantly follicular growth pattern but display nuclear features and overall clinical behavior consistent with PTC, even though, historically, FVPTC has been regarded as more aggressive compared with pure PTC (Li Volsi VA, 1994). Poorly differentiated (PDTC) and undifferentiated anaplastic carcinomas (ATC) seem to derive from the progression of differentiated carcinomas (Van der Laan BF, 1993). Although ATC represents 2-5% of thyroid malignant tumors, it is one of the most lethal human neoplasms being rapidly-growing, very aggressive and always fatal. Anaplastic cancers invade adjacent structures and metastasize extensively to cervical lymph nodes and distant organs such as lungs and bones. Finally, PDTCs, representing 7% of thyroid carcinomas, are morphologically and behaviorally intermediate between

well-differentiated and undifferentiated thyroid carcinomas. Medullary carcinomas of the thyroid are significantly less common than follicular cell-derived thyroid tumors and include multiple endocrine neoplasia 2A and 2B (MEN2A, MEN2B) and familial medullary thyroid carcinoma. This type of thyroid tumor is characterized by gain-of-function mutations of RET proto-oncogene.

2.3 Genetic lesions in thyroid carcinomas

2.3.1

Proto-oncogenes and oncogenes

The involvement of several oncogenes has been demonstrated in thyroid carcinomas (Figure 2). Mutations or rearrangements in genes encoding RET, NTRK1, BRAF or Ras are detectable in nearly 70% of all cases in well-differentiated thyroid carcinoma (Table 1) (Kondo T, 2006).

Genetic alteration	PTC	FTC	PDTC	ATC
RET rearrangement	13-43%	0%	0-13%	0%
Braf mutation	26-69%	0%	0-13%	10-35%
NTRK1 rearrangement	5-13%	Unknown	Unknown	Unknown
Ras mutation	0-21%	40-53%	18-27%	20-60%
TP53 mutation	0-5%	0-9%	17-38%	67-88%

Table 1. Genetic defects in papillary (PTC), follicular (FTC), poorly differentiated (PDTC) and anaplastic (ATC) thyroid carcinomas.

RET is a transmembrane receptor-tyrosine kinase encoded by RET proto-oncogene which is rearranged in sporadic and radiation-associated papillary carcinoma. The most common RET chimeric genes are H4(CCDC6)–RET (also known as RET/PTC1) and ELE1–RET (also known as RET/PTC3) in papillary carcinoma (Kondo T, 2006). The neurotrophic receptor-tyrosine kinase NTRK1 (also known as TRK and TRKA) is another proto-oncogene encoding a transmembrane tyrosine-kinase receptor for nerve growth factor rearranged in 5–13% of sporadic but only 3% of radiation-induced papillary thyroid tumors. TPM3, TPR and TFG are the main fusion partners in the chimeric oncogenes TRK, TRK-T1, TRK-T2 and TRK-T3 (Kondo T, 2006). BRAF is a serine/threonine kinase involved in cell proliferation pathways. BRAFV600E is the most common alteration in sporadic papillary carcinomas. BRAF mutations are found in 29–69% of papillary thyroid carcinomas, in up to 13% of poorly differentiated thyroid carcinomas and 35% of undifferentiated anaplastic thyroid carcinomas but not in follicular thyroid carcinomas (Kondo T, 2006). Ras mutated is one of the most common genetic lesions found in human tumors. Ras proto-oncogenes known as HRAS, KRAS and NRAS play a crucial role in thyroid tumorigenesis. A low incidence of Ras mutation was found in well-differentiated thyroid carcinoma whereas this phenomenon is more frequent in poorly differentiated and undifferentiated thyroid carcinoma, thus indicating its importance in thyroid tumor progression. The critical role of *ras* gene activation

in some thyroid cancer histotypes was confirmed by the induction of thyroid follicular carcinomas associated to lung metastasis following the injection of the Kirsten murine sarcoma virus into the thyroid gland of adult Fischer rats (Portella G, 1989). The important role of *ras* oncogenes in thyroid cancer was also demonstrated in human follicular and anaplastic carcinoma where the frequency of mutation in this gene is about 50% (Nikiforova MN, 2003). Finally, PAX8-PPAR- γ rearrangements which juxtapose the thyroid transcription factor PAX8 to the peroxisome proliferator-activated receptor (PPAR- γ) and impairment of the TP53 tumor suppressor gene were also reported in follicular and anaplastic carcinomas, respectively (Kroll TG, 2000; Suarez HG, 1990; Fagin JA, 1993). Very recently another activated form, called B-RAF, has been reported in irradiated patients of the Chernobyl area, consisting in the in-frame fusion of the first eight exons of the A-kinase anchor protein (AKAP9) gene with the carboxyl-terminal encoding-region (exons 9-18) of BRAF that results from a paracentric inversion [inv (7)q21-22q34] of the long arm of chromosome 7 (Fusco A, 2005).

2.3.2

Tumor suppressor genes

Tumor suppressor genes are normal genes that control cell division, repair DNA mistakes, and tell cells when to die. All these processes are required for the

normal cell differentiation, organogenesis and tissue homeostasis of all multicellular organisms. When tumor suppressor genes do not work properly, cells can grow out of control and eventually lead to cancer. The mechanisms that may alter the normal function of tumor suppressor genes are numerous. The first, and most important, model to explain how the function of a tumor suppressor gene may be invalidated was proposed by Knudson in the early 70s (Knudson AG, 1971) and provided a two-hit events for gene inactivation. Both copies of a tumor suppressor gene should be inactivated either through nonsense or truncating mutations. Nevertheless, studies conducted in multiple tumor types highlight a new generation of tumor suppressor genes that redefine Knudson's hypothesis to include epigenetic inactivation as one or both of the two hits required for tumor suppressor gene inactivation (Paige AJ, 2003). Among the most important and well studied inactivating epigenetic events, hypermethylation of CpG islands in the gene promoter region or in other regulatory regions and the lack of an allele (loss of heterozygosity, LOH) may account for a reduced expression of tumor suppressor genes, and as consequence, a decreasing of their function. Studies indicate that genetic and epigenetic events can work either in combination or in isolation to inactivate tumor suppressor genes in cancer.

In thyroid neoplastic lesions, despite the high frequency of oncogenes activation, tumor suppressor genes clearly involved in thyroid carcinogenesis still need to be

identified. Impairment of the p53 tumor suppressor gene function, overall through nonsense point mutations, represents a typical feature of anaplastic carcinomas (Fagin JA, 1993). Decreased levels of PTEN (Bruni P, 2000) and PTPRJ (Iuliano R, 2003), a dual specific phosphatase and a receptor tyrosine phosphatase, respectively, have been described in thyroid malignancies: both these proteins increase the stability of p27(KIP1), which in turn can negatively regulate the transition through the cell cycle. Therefore, it is possible say that the identification of other genes whose impairment of function might favor thyroid cell transformation, represents a prior aim in thyroid cancer research since, until now, very little is known about them.

2.4

The Cl 2 gene

Our group has recently identified (Visconti R, 2003), by a differential screening of PC Cl 3 rat cell lines versus the same cells transfected with the Adenovirus E1A (PC E1A), a gene named Cl 2, that was strongly upregulated in PC E1A cells. The rat Cl 2 transcript is 4.4 Kb long and encodes a 949 amino acid protein. In human thyroid neoplastic cell lines and thyroid carcinomas the corresponding orthologues gene was drastically down-regulated, even though the number of neoplastic samples analyzed in the study was not exhaustive. To explain the contrasting behavior of Cl 2 expression in PC E1A cell lines and

thyroid carcinoma, the effects of the E1A adenovirus infection on eukaryotic cells have been analyzed. Interestingly, it appears that the same E1A activities required for oncogenic transformation also stimulate programmed cell death. (Whyte P, 1988; Chinnadurai G, 1992; White E, 1995). Induction of apoptosis by E1A adenovirus may explain why the Cl 2 gene was overexpressed in PC E1 A cell lines. Consequently, based on the pro-apoptotic effects of E1A infection and on the finding that in human thyroid carcinoma cell lines and human thyroid tumors Cl 2 was down-regulated, the authors hypothesized that this gene might function as a tumor suppressor gene.

A schematic organization of human Cl 2 protein sequence is shown in Figure 2.



Figure 2. Human Cl 2 protein. In grey are indicated the protein segments without known motifs. Segment in red indicates a signal peptide; segments in pink indicate nuclear localization signals (NLS) ; segment in green indicates coiled coil region; segments in blue indicate regions containing repeats.

Bioinformatic analysis of protein sequence shows the presence of nuclear localization signals (NLS), Figure 2. These segments are scattered through the entire protein aa sequence 493 (PKKK), 550 (KKKK), 584 (KKKK), 73 (PLQRRRS) 492 (PPKKAQ), 493 (PKKKAQD), 530 (PLKKAKE), 546 (PEKEKKK), as well as bipartite NLS at aa 419 (RKDQHRERPQTTRPSK), 538 (KKHEKLEKPEKEKKKKM), 570 (KKSEKKSQKEKEKSKKK), 574

(KKSKQEKEKSKKKKGGK), 609 (KKSVDLLGSFEGKRRL), and 764 (KKQSLENFLSRFRWRRR) and are characterized by the presence of lysine-rich region.

The presence of a signal peptide (Figure 2) suggested that the protein may be secreted. Although the presence of the protein in the culture medium has been demonstrated in certain papers (Tremblay F, 2009; Okada T, 2008; Yi Liu, 2004; Hong Mu, 2003), a clear demonstration that CCDC80 may be present also in extracellular environment requires further investigation because other authors have verified that the localization of the protein was exclusively nuclear and/or cytoplasmatic (Bommer G, 2004).

Three regions of the protein (RPT, Figure 2) contain internal repeats. The three repeats represented the most highly conserved regions when the human sequence was compared with rat and mouse protein and showed an identity ranging from 91% to 98%. These three regions shared also a homology of about 30% with the carboxyl-terminal regions of the human genes “shushi-repeat protein on the X-chromosome” (SRPX) and “sushi repeat protein upregulated in leukemia” (SRPUL). The SRPX gene has been linked to the neoplastic transformation of fibroblast and was in fact expressed in normal rat cells, but completely suppressed in cells transformed by v-src (Pan J, 1996); whereas SRPUL was found normally expressed in heart, ovary, and placenta, but could not be detected in leukemic cell lines (Kurosawa H, 1999). Finally, a coiled coil domain is present between aa residues 547-587. Many coiled coil type proteins are

involved in important biological functions such as the regulation of gene expression, e.g. transcription factors. These domains are protein-protein interaction domains consisting of two or more alpha-helical motifs twisting around each other to form a supercoil (Lupas and Gruber, 2005). The genetic locus of Cl 2 is on chromosome 3, 3q13.2. The human protein is 108 kDa.

In a recent paper (Bommer GT, 2004) was reported the identification of a gene, named DRO1, that stands for “Down-Regulated by Oncogenes 1”, which was downregulated in rat RK3E epithelial cells neoplastically transformed by β -catenin oncogene, in colon and pancreatic cancer cell lines as well as in few cases of human colorectal tumors. These results suggested a possible central role of DRO1 in colorectal and pancreatic cancer. The DRO1 gene has a complete homology with the human orthologous gene that Visconti and colleagues identified in rat thyroid cell lines transfected with E1A adenovirus. Currently, in the NCBI database this gene is recorded with the name of Coiled-Coil Domain Containing 80 (CCDC80) but it is also known as URB, DRO1, SSG1, okuribin, MGC131805, MGC134851.

Marcantonio M. and colleagues (Marcantonio M, 2001) reported the identification of a gene named “steroid-sensitive gene 1” (SSG1) that was regulated by estrogen and involved in rat mammary gland cancerogenesis. Although they reported the analysis of a protein of only 40-kDa, the open reading frame (ORF) cloned by them was identical to the ORF of rat Cl 2 studied

by Visconti R. and colleagues. The authors treated intact rats with estrogens and then analyzed the mRNA level of SSG1 gene in total RNA from the mammary glands and uterus. In mammary gland northern blot indicated high level and no significant regulation of SSG1 mRNA in E₂-treated samples versus the control group, despite down-regulation in the uterus. Corresponding protein samples were extracted from mammary gland and uterus tissues and immunoblot was performed using an affinity purified anti-SSG1 antibody. In spite of the high levels of SSG1 mRNA, SSG1 protein was very low or undetectable in control untreated mammary tissue. In contrast, SSG1 protein expression was significantly increased by 16-fold in E₂-treated rats. Because SSG1 protein levels in 7,12-dimethylbenz(*a*)anthracene-induced rat mammary tumors were 23-fold greater than SSG1 levels in resting mammary tissue, and 8-fold higher than protein levels expressed in lactating mammary glands, the author assumed that SSG1 plays a role in estrogen functions, and its overexpression is correlated with mammary carcinogenesis.

Cl 2 has been shown to be upregulated in brown adipose tissue of mildly obese bombesin receptor subtype-3 (BRS-3)-deficient mice (Aoki K., 2002). In this work the author named the gene Urb (Up Regulated Bombesin) and suggested that it may have a unique function in the regulation of body weight and energy metabolism.

Involvement of Cl 2 in embryogenesis has been proposed by Yi Liu and

colleagues studying mouse development. They supported this role mainly by the evidence of a temporal expression profile detected in whole mouse embryos of various stage using northern blot analysis in which the Cl 2 expression increased with the progression of embryogenesis (Yi Liu, 2004).

Further functions have been proposed for Cl 2 in chicken were it was known as Equarin (Hong Mu, 2003). In this work it was shown that microinjection of equarin mRNA into *Xenopus* embryos induced abnormal eye development. The authors suggested that equarin was involved in eye formation.

Finally, in very recent papers the functions of Cl 2 in the adipose tissue and in adipogenesis were analyzed (Okada T., 2008; Tremblay F., 2009). Okada and colleagues investigated the involvement of the gene in the adipose tissue homeostasis and in obesity. They found that Cl 2 mRNA was predominantly expressed in adipose tissue and was downregulated in obese mouse models and in diet-induced obese mice. Similar results were confirmed in vitro using 3T3L1 adipocytes cell lines treated with, insulin, TNF- α , H₂O₂ and hypoxia which caused a reduction of URB mRNA level. Tremblay and colleagues demonstrated that Cl 2 is required for the full inhibition of T-cell factor-mediated transcriptional activity, down-regulation of Wnt/beta-catenin target genes during clonal expansion and the subsequent induction of C/EBPalpha and peroxisome proliferator-activated receptor gamma (PPAR γ). Surprisingly, the author found that overexpression of Cl 2 in 3T3-L1 cells also inhibits adipocyte differentiation

without affecting the repression of the Wnt/beta-catenin signaling pathway. Therefore, they suggested that Cl 2 plays dual roles in adipogenesis by mechanisms that involve, at least in part, down-regulation of Wnt/beta-catenin signaling and induction of C/EBPalpha and PPAR γ .

To validate the potential role of Cl 2 gene in the onset of tumors and in particular in thyroid carcinogenesis, in this experimental thesis I have evaluated its expression by a quantitative RT-PCR in a larger number of thyroid neoplastic samples, and investigated the effects of the restoration of Cl 2 in two human thyroid carcinoma cell lines which expressed very low level of this gene. In these cell lines I measured proliferation, ability to grow in a semisolid medium and invasion potential. In addition, a knock out mouse model for Cl 2 gene was generated in order to study the effect of the loss of Cl 2 also in vivo.

I observed that Cl 2 gene expression was drastically downregulated in thyroid carcinomas, particularly in the follicular variant of the papillary carcinomas. Consistently, these last samples showed a quite high percentage of LOH at the Cl 2 locus. Furthermore, the restoration of Cl 2 expression led to the growth inhibition of thyroid carcinoma cell lines and to the impairment of their ability to grow in soft agar. Finally, the mice null for the Cl 2 gene developed thyroid adenomas, ovary carcinomas and when crossed with RET/PTC1 transgenic mice the effects on thyroid gland were impressive. This data suggested a critical role of the loss of Cl 2 expression in thyroid carcinogenesis.

3

Materials and methods

Collection of neoplastic tissues

Thyroid, colon, ovary and breast tumors were collected at the Service d'Anatomo-Pathologie, Centre Hospitalier Lyon Sud, Pierre Benite, France. Neoplastic human tissues and normal adjacent tissue or the controlateral normal thyroid lobe were obtained from surgical specimens and immediately frozen in liquid nitrogen. The tumor samples were stored frozen until RNA extractions were performed.

RNA and DNA extraction

Total RNA isolation from human tissues and cell lines was performed with Trizol (Invitrogen) according to the manufacturer's instructions. RNA was extracted from fresh specimens after pulverizing on liquid nitrogen using stainless steel mortar and pestle. The integrity of the RNA was assessed by denaturing agarose gel electrophoresis. To ensure that RNA samples were not contaminated with DNA, negative controls were obtained by performing the PCR on samples that were not reversed-transcribed but otherwise identically processed.

DNA was prepared for each sample from a portion of liquid nitrogen pulverized

tissue using QIAamp DNA Mini Kit (QIAGEN group) following the Tissue Protocol of instruction manual.

Real-time PCR

cDNA preparation. 1 µg of total RNA of each sample was reverse-transcribed with the QuantiTect[®] Reverse Transcription (QIAGEN group) using an optimized blend of oligo-dT and random primers according to the manufacturer's instructions.

Selection of primers and probes. To design a qRT-PCR assay I used the Human ProbeLibrary[™] system (Exiqon, Denmark). Using ProbeFinder assay design software I chose the best probe and primers pair. All fluorogenic probes were dual-labeled with FAM at 5' end and with a black quencher at the 3' end. I chose probe and primers to amplify a fragment for real-time PCR of Cl 2 mRNA, entering its accession number (NM_199511.1) on the ProbeFinder software. I chose an amplicon of 60 nucleotides scattered between 5th and 6th exons. The number of probe was "human 25" (according to the numbering of Exiqon's Human ProbeLibry kit) and the primer sequences were: Cl 2 forward 5'-tcctggagaacttcctatcc-3'; Cl 2 reverse 5'-agcagagatcaccagcaacc-3'. The same procedure was used to choose both probe and primers for the housekeeping gene G6PD, accession number X03674, and for Beta-catenin gene accession number NM_001904.3. For G6PD I opted for an amplicon of 106 nucleotides scattered

among 3th and 4th exons. The number of probe was “human 05” (according to the numbering of Exiquon’s Human ProbeLibry kit) and the primer sequences were: G6PD forward 5’-acagagtgagcccttcttcaa-3’; G6PD reverse 5’-ggaggctgcatcatcgact-3’. For Beta-catenein I chose an amplicon of 90 nucleotides scattered among 6th and 7th exons. The number of probe was “human 08” (according to the numbering of Exiquon’s Human ProbeLibry kit) and the primer sequences were: B-catenin forward 5’-tgtaaattcttggtattacgaca-3’; B-catenin reverse 5’-ccaccactagccagtatgatga-3’.

Relative Quantitative. TaqMan qPCR was performed in 7900HD SDS machine (Applied Biosystem, USA) in 384-well plates using a final volume of 20 µl. For PCR I used 8 µl of 2,5x RealMasterMixTM Probe ROX (Eppendorf AG, Germany) 200 nM of each primer, 100 nM probe and cDNA generated from 50 ng of total RNA. The conditions used for PCR were 2 min at 95°C, and then 45 cycles of 20 sec at 95°C and 1 min a 60°C. Each reaction was performed in duplicate. To calculate the relative expression levels I used the $2^{-\Delta\Delta CT}$ method (Kenneth J, 2001).

Immunohistochemical analysis

Paraffin sections (5-6 µm) were deparaffinized, placed in a solution of absolute methanol and 0.3% hydrogen peroxide for 30 min and then washed in PBS before immunoperoxidase staining. For Cl 2 immunostaining, I used a

commercial polyclonal antibody (anti-URB) purchased by R&D Systems Inc. (Minneapolis, USA) produced in goats immunized with the human purified Cl 2 peptide (Glu 22 - Tyr 650) fused with a 6X histidine tag at the carboxyl-terminus. The peptide used to produce antibody was sold by the same company. The slides were subsequently incubated with biotinylated anti-goat IgG for 20 min (Dako LSAB2 System) and then with streptavidin horseradish peroxidase for 20 min. For immunostaining the slides were incubated in diaminobenzidine (DAB-DAKO) solution containing 0.06 mM DAB and 2 mM hydrogen peroxide in 0.05% PBS pH 7.6 for 5 min. After chromogen development, the slides were washed, dehydrated with alcohol and xylene, and mounted with coverslips using a permanent mounting medium (Permount). No staining was also observed when the normal thyroid tissues were analyzed after the Cl 2 specific antibodies had been pre-incubated with the Cl 2 control peptide (data not shown). Micrographs were taken on Kodak Ektachrome film with a photo Zeiss system

Loss of heterozygosity (LOH) analysis

Five Single Nucleotide Polymorphisms (SNP) that showed high average heterozygosity levels, to obtain the highest number of informative cases, were chosen using the SNP database of NCBI. To identify the SNPs scattered in the genetic locus of Cl 2 I used the putative sequence of URB human gene. The SNPs reference, alleles, primers and annealing temperatures used to perform

PCR are listed in Table 2. The primers used for PCR were also used for sequencing assays.

SNP A.N.	Alleles	Primer forward	Primer reverse	Annealing T.
rs4340680	A/C	5'-catttacatattgtagcccccac-3'	5'-cacattctaattgtagttcaagttgtc-3'	58°C
rs7349537	A/T	5'-gctacctctcttgattaccctc-3'	5'-ggaaagtcacagcaagcatg3'	57°C
rs10511316	T/C	5'-agctcatccagatcaggctg-3'	5'-tttcactactagccagcaccag-3'	58°C
rs981181	A/C	5'-gaaccacttgaattacaggag-3'	5'-gaggttgacgatagtgagac-3'	56°C
rs3842905	C/G	5'-ccaacaaggactccttctactc-3'	5'-tggcaaaagagggcagcag-3'	58°C

Table 2. SNP Accession numbers, alleles genotypes, primers and annealing temperature used to carry out the LOH analysis on human Cl 2 genomic locus

Briefly, genomic DNA was PCR amplified in a region spanning about 400 bp around the SNP analyzed, then the purified PCR product was sequenced. I measured the height of the two peaks on the chromatogram and calculated the ratio of the two alleles in the matched tumor/normal samples: LOH was defined if the ratio in the carcinoma sample was <50%.

PCR

PCR was performed using HotMaster Taq DNA Polimerase (Eppendorf AG, Germany) in a final volume of 25 µl. For amplification reaction I used 50 ng of genomic DNA, 0,5 unit of Hotmaster Taq DNA polymerase, a final concentration of each primer of 0.2 µM and 0.2mM of dNTPs and 2,5 µl of 10x HotMaster Taq DNA Polimerase Buffer with Mg²⁺. The conditions used for PCR was an initial denaturation at 94°C for 2 min, 35 cycles of 94°C for 20 sec, 56°C-

58°C for 10 sec, 70°C for 40 sec and a final extension of 5 min at 70°C. After amplification, size and quality of amplicons were checked loading 5 µl of reactions on agarose gel. 20 µl of each PCR was sequenced with specific forward and reverse primers used for amplification reaction.

DNA methylation analysis

Sodium bisulphite conversion of genomic DNA (about 2 µg for each conversion) was obtained using Epitect[®] Bisulphite kit (QIAGEN group) following the purchaser's instructions.

PyrosequencingTM analysis: Quantitative DNA methylation analysis was performed using the PSQ 96MA instrument from Pyrosequencing (Biotage AB, Uppsala, Sweden) following the protocol suggested by the manufacturer. Primers used for PCR reactions were: Cl 2 F1 (position from nucleotides -102 to -79) 5'-ttggaaggggtaaggtattagtt -3' (5'-Biotinylated); Cl 2 R1(from +89 to +112) 5'-aactcaacaacaacaaccactaa -3'. Amplification were carried out on 10 ng of bisulphite treated DNA using HotStarTaq DNA polymerase (QIAGEN group) under the following conditions: 15 min at 95°C, followed by 50 cycles of 30 sec at 95°C, 40 sec at 53,4°C, and 1 min at 72°C, then a final elongation of 10 min at 72°C before holding at 4°C, in a final reaction volume of 50 µl. PCR final products (214 bp) were then used for Pyrosequencing reactions; sequencing primer (Cl 2 S1) was 5'-acaaatcaacaaaacacc-3'.

Bisulphite genomic sequencing: 2 µl of each sample were used as template in PCR reactions using the following primers: Cl 2 seq F1 5'-gtggattatataatgatatggagaatggg-3' and Cl 2 seq R1 5'-cccctaactactaatctttactcaaac-3' for the analysis of Cl 2 CpG islands, which are located on the second **exon**. Amplifications were carried out on 10 ng of bisulphite treated DNA using the same protocol described above. PCR final products were then cloned into the pGEM-T-easy vector provided by Promega pGEM[®]-T-Easy Vector System II (Promega Italia, Milan, Italy) following the supplier's procedures. The purified plasmids were sequenced in both directions using T7 and Sp6 primers. At least 20 independent clones were sequenced to determine the methylation pattern of individual molecules.

Assay of the transformed state

Soft agar assays were performed according to a previously described technique (Montagnier L, 1964). The tumorigenicity of the cell lines was tested by subcutaneous injections of 2×10^6 cells into athymic mice. The animals were monitored at regular intervals for the appearance of tumors.

Generation of Cl 2-KO mice

We used gene targeting techniques in embryonic stem (ES) cells to generate a

null mutation at the murine Cl 2 genomic locus. We deleted exon, which contains the start site of the murine Cl 2 gene and replaced them with a neomycin-resistant gene. Heterozygous progeny of chimeric animals were identified by Southern-blot analysis of EcoRI-digested tail DNA (data not shown), and mutants were established to produce mice heterozygous or homozygous for the Cl 2-null allele. Both heterozygous and homozygous mutant mice were viable and fertile. All mice were maintained under specific pathogen-free conditions in our Laboratory Animal Facility (Istituto dei Tumori di Napoli, Naples, Italy) and all studies were conducted in accordance with Italian regulations for experimentations on animals.

Cell cycle analysis.

Cells were harvested in PBS containing 2 mmol/L EDTA, washed once with PBS, and treated for 30 min in cold ethanol (70%). Cells were washed once in PBS and permeabilized with 0.2% Tween 20 and 1 mg/mL RNase A for 30 min, and washed and stained with 50 µg/ml propidium iodide. Stained cells were analyzed with a FACSCalibur (Necton Dickinson), and the data was analyzed using a Mod-Fit cell cycle analysis program.

Generation of CL 2 stable clones

Thyroid carcinoma cell lines (FRO and KAT-4) were transfected using Arrest-in

reagent (Open Biosystems, Huntsville USA) with p3XFLAG-CMV.10 plasmid (Sigma, Saint Louis USA) carrying the human full-length coding sequence of Cl 2 gene (pCMV.10-Cl 2/CCDCD80), and with empty vector. Briefly, I transfected 10 µg of plasmids in a 100 mm cell culture plate using a ratio DNA/Arrest-in of 1:5 following the manual's instruction of Arrest-in. The transfected cells were selected in a medium containing geneticin 1250 µg/ml (G418; Life Technologies). For each transfection, several G418-resistant clones and the mass cell population were isolated and expanded for further analysis. For Transient transfection I used the same transfecting agent and the same protocol described above.

Generation of mouse embryonic fibroblasts (MEFs)

MEFs have been established from wild-type, CL 2^{+/-} and CL 2^{-/-} embryos 12.5 days *post coitum*. Briefly, after removal of the head and internal organs, embryos were rinsed with phosphate-buffered saline (PBS), minced, and resuspended in DMEM containing 10% FBS and 2 mM glutamine, and 100 IU/ml penicillin and 100 µg/ml streptomycin following standard procedures. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (fetal bovine serum), glutamine, penicillin and streptomycin in a 5% CO₂ atmosphere.

Invasion quantification assay

Invasion assays were performed using 24-well transwell units with 8- μ m polycarbonate Nucleopore filters (Corning, NY). 2×10^4 of Cl 2 stable clone cells and the same number of FRO cells were resuspended in 100 μ l of medium with and without serum and placed in the upper compartment, and 600 μ l of medium with serum were added in the lower compartment. The transwell plates were then incubated at 37° for 48h in a humidified atmosphere with 5% CO₂. Plates were then rinsed with PBS, and non invading cells retained in the upper surface of the membrane were removed by a cotton swab. Crystal violet solution was used to stain invading cells, located in the lower surface of the membrane. To quantify the cells retained by polycarbonate Nucleopore filters both in control and in stable clone, I removed the crystal violet from Nucleopore filters using a 1% SDS solution and measured the absorbance at 575 nm and calculate the ratio: $(ABS_{575} \text{ FRO-CloneB} / ABS_{575} \text{ FRO cell}) \times 100$.

Directional cell migration in vitro wound healing

A cell monolayer was cultured until a 85-90% of confluence, respectively for FRO-Cl 2 cell and control cells in 60 mm cell culture disks. Using a pipette tip I disrupted the cell layer mechanically in order to obtain a section of cell culture disk without cells. After the wound, I captured the images at the beginning and

after regular intervals during cell migration. I stopped to photograph the plate after 20 hours because the different mitotic activity and cell growth speed, among FRO-Cl 2 stable clone and FRO control cells. clone, could distort the data.

Identification of apoptosis TUNEL assay

For the detection of apoptosis I used In Situ Cell Death Detection Kit, TMR red (Roche Applied Science Germany) based on TdT-mediated dUTP nick end labelling (TUNEL) technique. 25×10^4 FRO cells, for each well, were transiently transfected in a 6-well plate with pCMV.10-Cl 2 construct, empty vector and with a vector carrying the EGFP protein to estimate the transfection efficiency. After 48h from transfection I estimated the transfection efficiency in 40% and started the procedure for apoptosis detection following the manual's instructions.

RESULTS

4.1

Cl 2 expression is drastically reduced in human thyroid carcinomas.

A previous analysis of a limited number of human thyroid neoplastic samples showed a reduced Cl 2 gene expression in most of the papillary thyroid carcinomas (PTC) and in some follicular adenomas (Visconti R., 2003). However, for the final assessment of the Cl 2 mRNA expression levels in human thyroid neoplasias, I analyzed the Cl 2 expression in a larger number of thyroid neoplastic samples including 7 goiters, 10 adenomas, 36 classic papillary carcinomas, 33 follicular variants of papillary carcinomas, 8 follicular carcinomas, and 8 anaplastic carcinomas by a quantitative RT-PCR assay, as described in the Materials and Methods section. The results are shown in Figures 2 and 3. I placed a cut-off value of ± 2 fold to assign an up- or downregulation. The variations between -2 and +2 fold with respect to the controls have been considered physiological. With this assumption, Cl 2 expression was significantly downregulated (≤ 2 fold) in 83.5% (71 out of 85) of carcinomas with respect to the normal thyroid tissue. In particular, a drastic reduction in Cl 2 gene expression was detected in the follicular variant of papillary carcinomas with an average of -54.5 fold. In 8 out of 33 of these neoplasias the fold change was less than 60, and in some cases the expression of Cl 2 was almost undetectable. In

contrast, the reduction in Cl 2 gene expression was less drastic in the classic forms of papillary carcinomas and in anaplastic thyroid carcinomas with an average fold-change of -11.4.

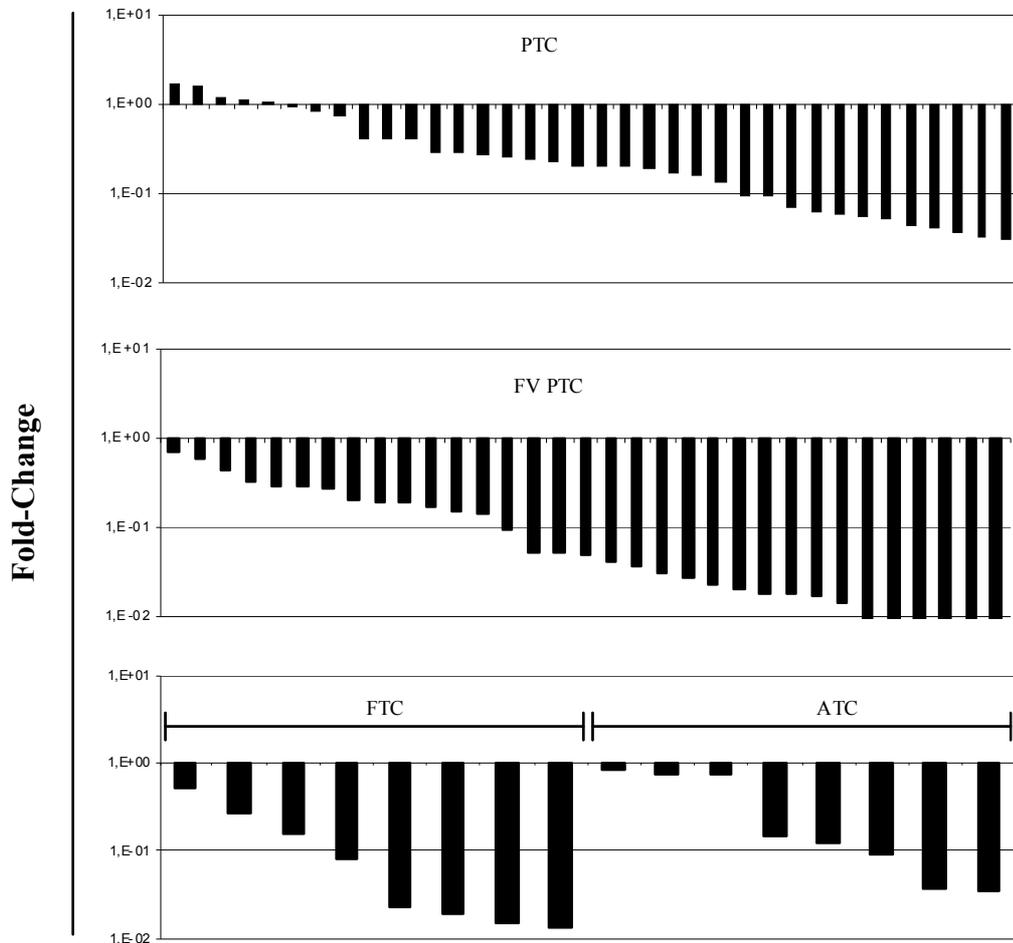


Figure 2. Quantitative PCR on thyroid tumors. Samples are divided based on their histotype. Papillary Thyroid Carcinomas (PTC), Follicular Variant of PTC (FV PTC), Follicular Thyroid Carcinomas (FTC), Anaplastic Thyroid Carcinomas (ATC). Values are expressed in fold-change in a logarithmic scale with respect to a pool of normal samples which equaled 1.

All papillary carcinomas analyzed in this study were also screened for the presence of the known genetic alterations present in PTCs, i.e. RET/PTC 1 and RET/PTC3, B-RAF mutations, TRK-T1 and ras gene family mutations. In Figure 4 I show the expression of Cl 2 in the PTCs carrying the activation of

RET and TRK-T1 and mutations in the B-RAF gene.

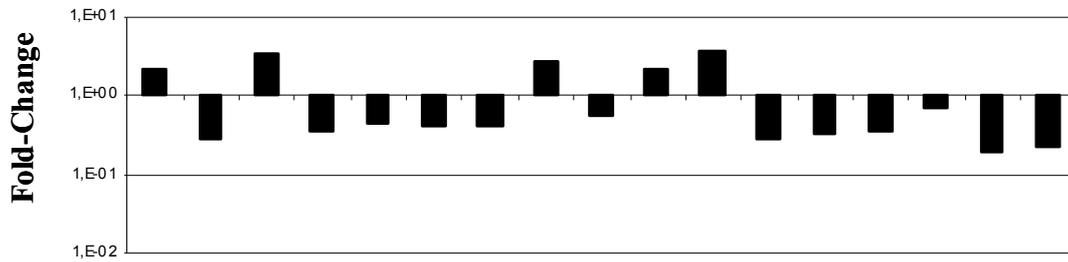


Figure 3. Expression analysis on thyroid goitre and adenoma of Cl 2 mRNA. There were no considerable variations in gene expression in this kind of benign lesions.

Carcinoma samples mutated in B-RAF have a behavior quite similar to the samples where the typical genetic alterations for PTCs were not detected, with an average fold-change decrease of 4.27. Conversely, the PTCs carrying the RET/PTC1 rearrangement did not show a significant downregulation of Cl 2 expression. Interestingly, Cl 2 was drastically downregulated in the only 2 PTCs carrying RET/PTC 3 rearrangements with a fold change of -75,5.

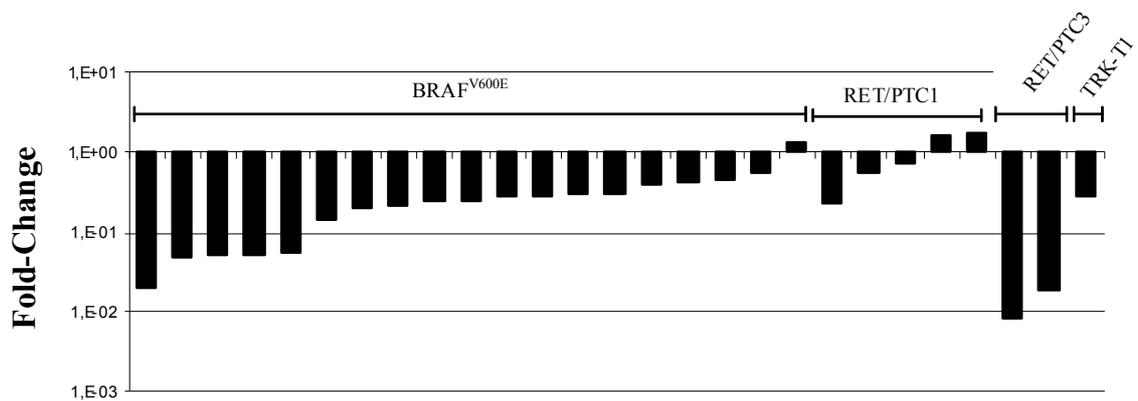


Figure 4. Expression analysis of Cl 2 mRNA correlated to oncogenes in thyroid carcinomas tumor. The average value of down-regulation in BRAF^{V600E} group was -4.27, in RET/PTC1 group was -1.98, in RET/PTC3 75.5 and the unique sample that presented a TRK-T1 rearrangement the value was -3.72.

4.2

Analysis of loss of heterozygosity (LOH) at the Cl 2 locus (3q13.2) in thyroid neoplastic samples.

Loss of one allele often accounts for reduced gene expression. Therefore, the thyroid neoplastic samples were analyzed for LOH at the Cl 2 locus. I used 5 SNPs markers to evaluate LOH on chromosome 3 at the Cl 2 locus in 70 cases of thyroid carcinomas of different histotypes.

After the screening of the informative and uninformative cases, I selected 45 informative cases and the results are summarized in Table 3. The loss of heterozygosity at the Cl 2 locus was observed in 16 cases (35,5 %) with the highest frequency in the follicular variant of papillary carcinomas (10/20; 50%). A lower frequency was observed in the classic PTCs (3 out of 13; 23%). Among the 5 informative cases of anaplastic carcinoma samples I observed LOH in 3 cases: a higher number of cases should be analyzed to have a reliable frequency of Cl 2 LOH in this histotype. Conversely, no LOH at the Cl 2 locus was observed in the follicular adenoma analyzed (0/6).

Diagnosis	Info.cases	LOH	%
Adenoma	7	0	0
Papillary Carcinoma	13	3	23
Follicular Variant of Pap. Carcinoma	20	10	50
Anaplastic Carcinoma	5	3	60
Total	45	16	35,5

Table 3. LOH analysis. 35,5% of human thyroid carcinomas samples analyzed showed an LOH event on genomic locus of Cl 2

When I compared C1 2 expression of the follicular variants of papillary carcinomas samples showing or not LOH presence, I found that C1 2 expression was much lower in the cases with LOH ($p=0,0011$) (Figure 5). Since it has been recently reported (Bommer GT, 2005) that C1 2 gene expression is down-regulated by beta-catenin, I analyzed the expression of beta-catenin in the thyroid tumour samples. As shown in Figure 6, an increased beta-catenin expression was detected in all thyroid carcinomas.

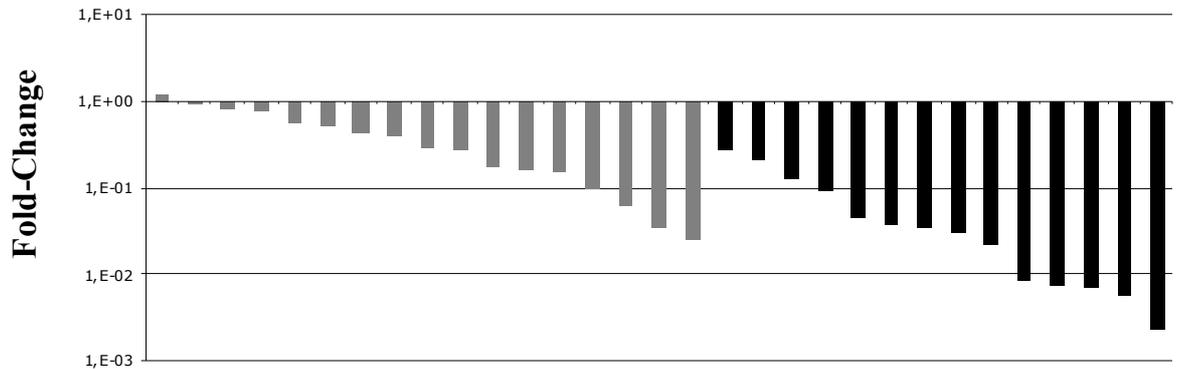


Figure 5. Correlation of expression levels of C1 2 between thyroid carcinomas samples without LOH (grey bars) and samples with LOH (black bars). The samples with LOH event present a greater reduction of C1 2 mRNA.

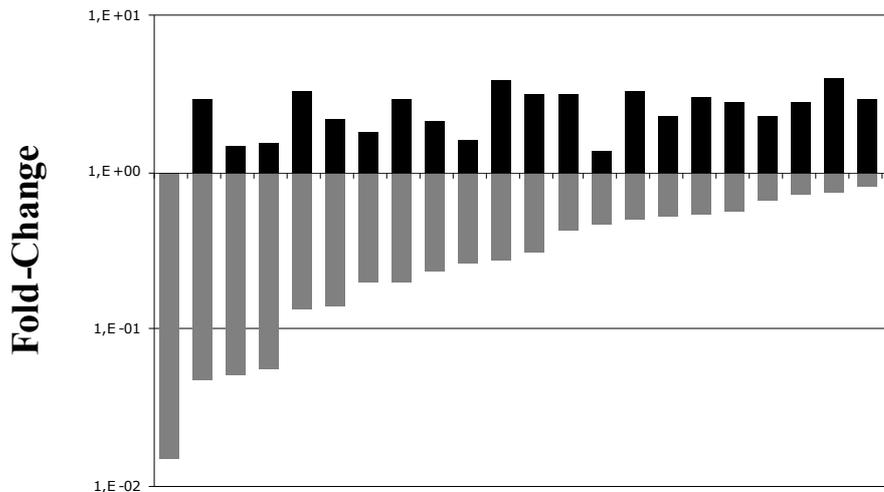


Figure 6. Each histogram reports the expression value of C1 2 (grey bars) and beta-catenin (black bars) in the same sample.

However, in the samples analyzed there is no significant proportional correlation between increase of beta-catenin expression and Cl 2 downregulation, apart from few cases.

4.3

Methylaton analysis of Cl 2 gene.

I investigated whether epigenetic mechanisms may contribute to Cl 2 gene silencing in thyroid tumors. The analysis of the genomic region surrounding the Cl 2 transcriptional start site (TSS), revealed that this region was very poor in CpG sites and the closest CpG islands were located about 1200 bp downstream the TSS, within the second exon (Figure 7).

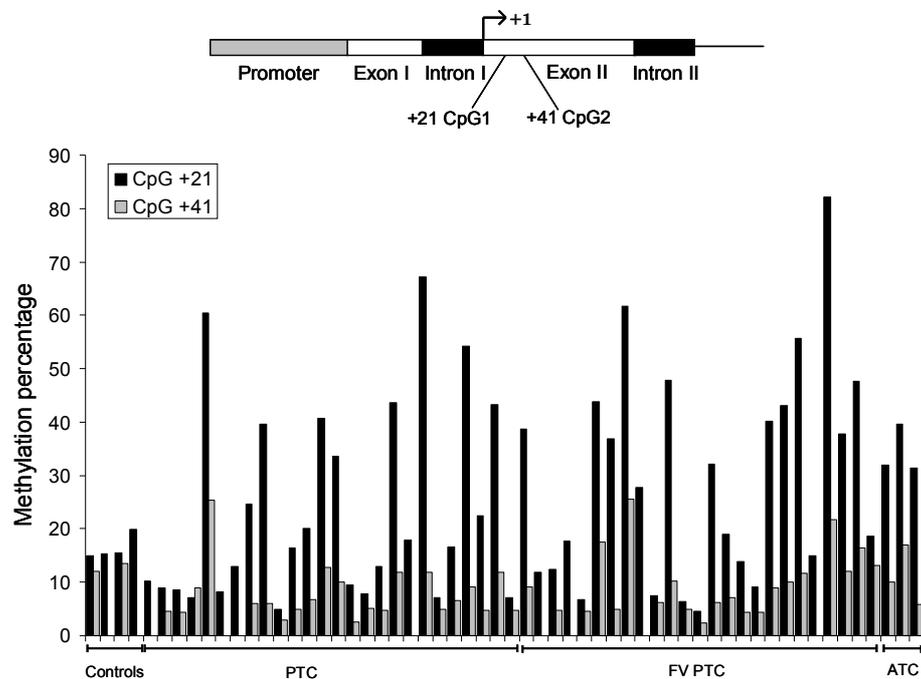


Figure 7. Methylation analysis of two putative regulatory CpG islands of Cl 2 gene. In the CpG +21 revealed a hyper-methylation in several samples respect to controls. Samples were divided base on the histotype. PTC, papillary thyroid carcinoma; FV PTC, follicular variant of PTC; ATC, anaplastic thyroid carcinomas.

However, I decided to analyze the methylation degree at both regions. The two CpG sites located just downstream the TSS (positions +21 and + 41) were analyzed for DNA methylation using the quantitative pyrosequencing technology. The results, presented in Figure 7, showed that the methylation degree of CpG site +21 was significantly higher in 42,5% (23/54) of tumor samples compared to normal thyroid tissue. Methylation levels of CpG site +41 were low and comparable among all the samples irrespective of their origin. Furthermore, by bisulfite genomic sequencing, 12 CpG sites located in the region +1242/+1543 within the downstream CpG island have been analyzed. This analysis showed that the average methylation of these sites was quite high (60%) but did not vary among the samples irrespective of gene expression and tissue origin (data not shown). Overall, this data suggests that the methylation state of CpG site +21 may contribute and represent a mark of Cl 2 gene silencing.

4.4

Immunohistochemical analysis of Cl 2 gene expression

Detection of Cl 2 protein by immunohistochemical analysis allows a rapid and sensitive screening of thyroid pathological tissues and is amenable to regular use as a routine diagnostic test. This technique was therefore chosen for Cl 2 protein analysis using a commercial antibody against a Cl 2 specific peptide.

The results of the immunohistochemical study of 44 thyroid specimens are summarized in Table 4. Not only did they confirm a downregulation of Cl 2 expression but also a translocation (nuclear > cytoplasm) in thyroid carcinoma, particularly in the follicular variant of PTC and in ATC.

A positive nuclear signal was detected in normal thyroid, goiter and adenoma (Figure 8 A, B, C) whereas no nuclear staining was observed in papillary carcinoma (Figure 8 D) and in follicular carcinoma (Figure 8 D) and anaplastic carcinoma (Figure 8 E). In the last two cases the signal was predominantly cytoplasmic but the intensity was lower with respect to normal thyroid. In anaplastic carcinoma the signal of Cl 2 was lost in 6 out of 10 samples (Table 4). For each case, sections were stained without the primary antibody and in all cases these controls were negative (data not shown).

Histotype	Samples	Negative	Positivity		
			1+	2+	3+
Normal thyroid	2			1 N>>C	1 N>C
Goiter	7	1	2 N>C	2 N	1 N 1 N>C
Adenoma	7	1	3 N	2 N	1 N 1 N/C
PTC	10	1	2 C	4 C	3 C
FTC	10	3	4 C	2 C	1 C
ATC	10	6	1 C	3 C	

Table 4. Results of immunohistochemical analysis. Samples were classified as negative when there was no staining with antibody anti-Cl 2, whereas for positivity has been used a scale with different intensity: 1+ weak, 2+ medium, 3+ strong. C and N indicate cytoplasmic and nuclear localization, respectively. When a subcellular translocation of the signal was present, it was indicated with >.

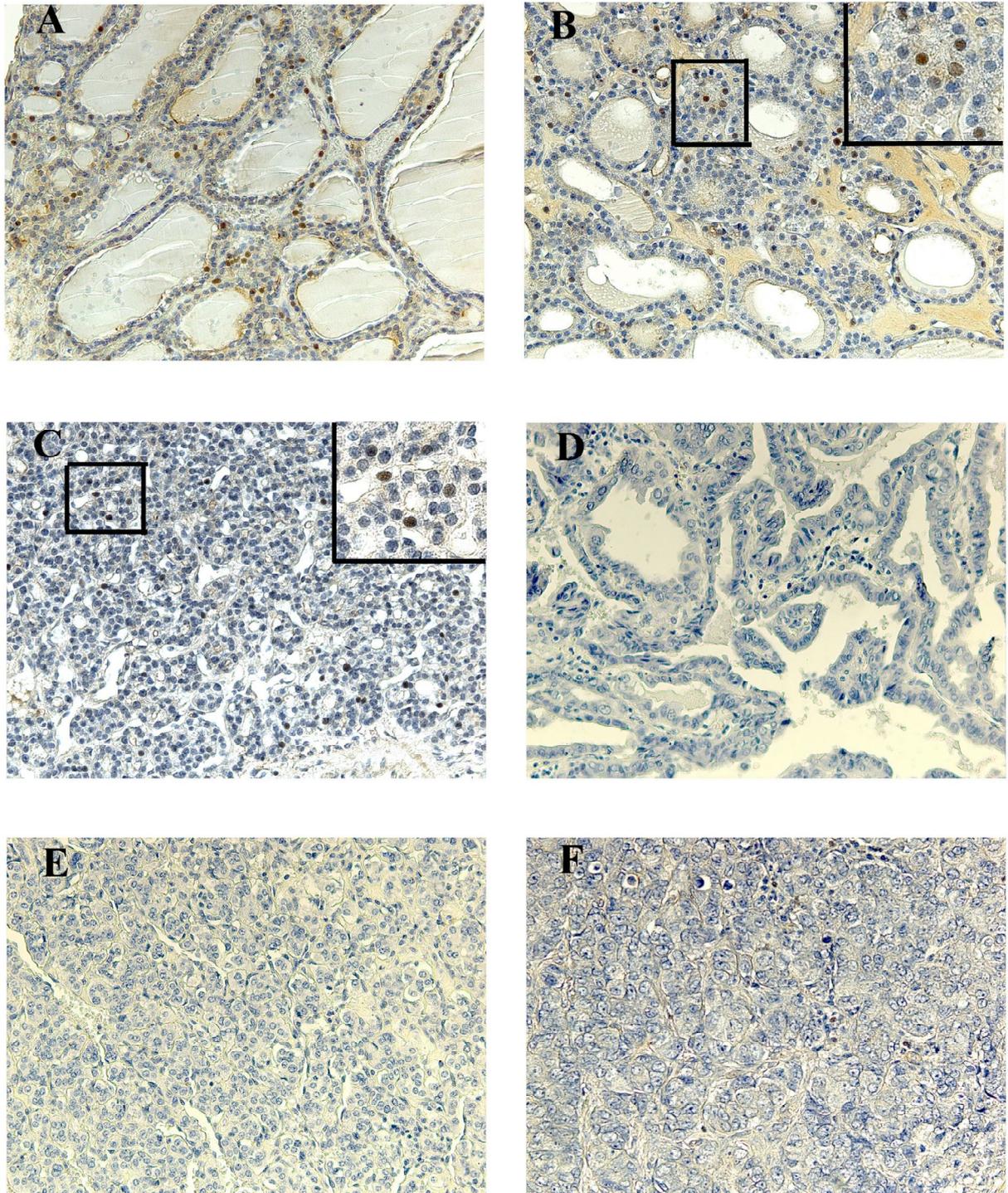


Figure 8. Immunohistochemical analysis of Cl 2 protein expression in benign and malignant thyroid tissues. Panel A: normal thyroid (200 x), showing nuclear immunoreactivity Panel B: Goiter (200 x), showing nuclear immunoreactivity, (inset shows 400 x magnification). Panel C: Thyroid adenoma (200 x), also in this case, a nuclear positivity was observed, (inset shows 400 x magnification). Panel D: Thyroid papillary carcinoma (200 x), negative for protein expression. Panel E: Thyroid follicular carcinoma (200 x), showing absent of Cl 2 protein expression. Panel F: An anaplastic thyroid carcinoma (200 x), showing no Cl 2 protein expression

4.5

Cl 2 expression is also downregulated in ovary, colorectal and breast carcinomas

The next step of my work has been to verify whether the downregulation of Cl 2 expression is restricted to thyroid carcinomas, or is a more general event in the process of carcinogenesis. As shown in Figure 9, Cl 2 appears drastically downregulated in the large majority of ovary, breast and colon carcinomas. In particular, no expression at all was detected in 10 out of 20 ovary carcinomas. These results would suggest that the loss of Cl 2 expression might also have a role in the development of these human carcinomas.

4.6

Restoration of Cl 2 gene expression reverts the malignant phenotype of thyroid carcinoma cell lines.

To determine whether the loss of Cl 2 gene expression affects thyroid carcinogenesis, I performed some functional analysis on two thyroid carcinoma cell lines, expressing very low level of mRNA of Cl 2, in which the expression was restored. To this aim I first carried out a growth curve on FRO cells stable expressing the Cl 2 gene (FRO-Cl2-B and FRO-Cl2-E) and, as shown in Figure 10, they grew with a significantly slower rate in comparison to the untransfected (FRO) and p3XFLAG-CMV.10 transfected cells (FRO E.V.).

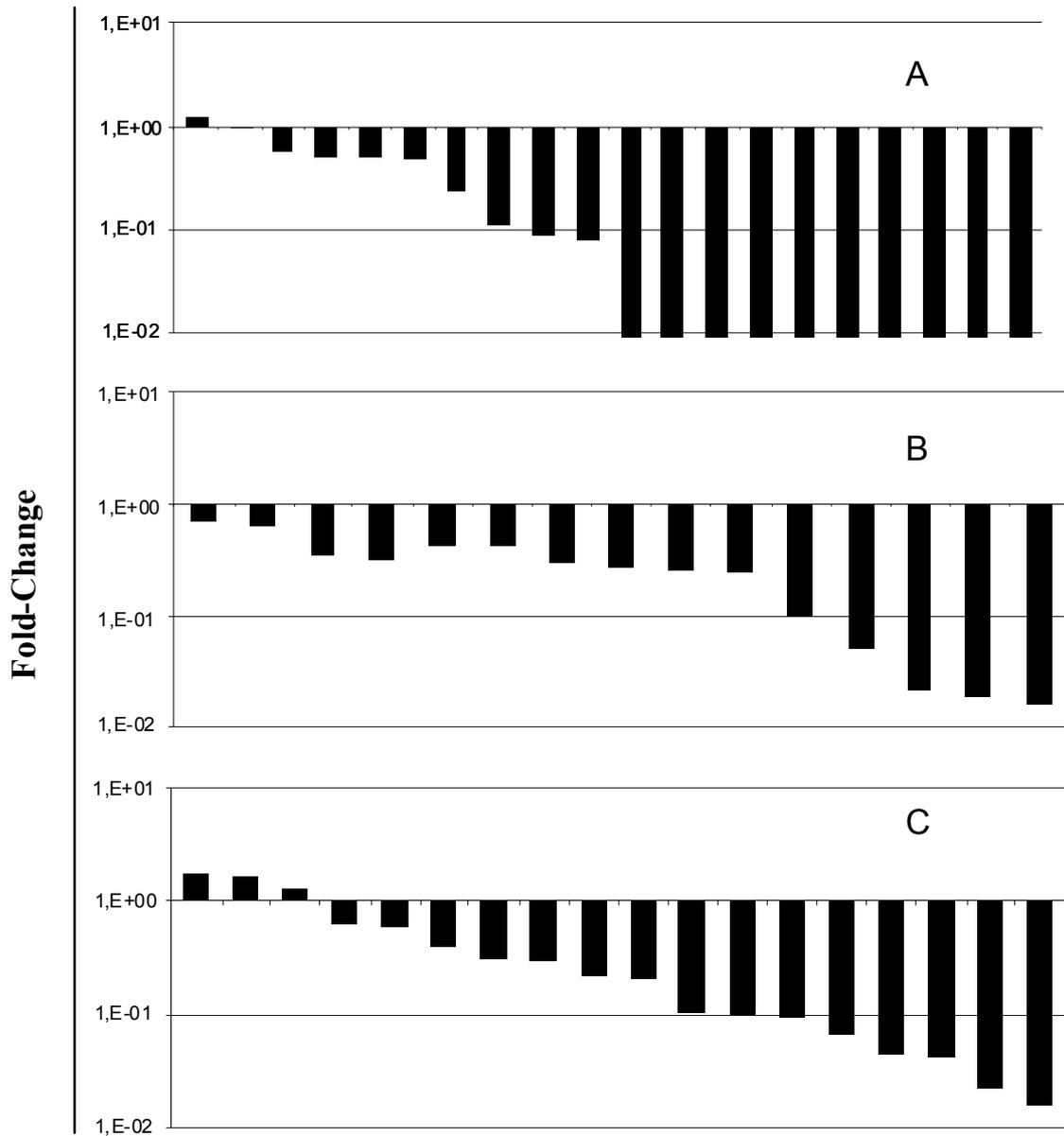


Figure 9. Expression analysis on Ovary (A), Colorectal (B) and Breast (C) carcinomas of Cl 2 mRNA. Values are expressed in fold-change in a logarithmic scale with respect to a pool of normal samples which equaled 1.. In panel A value of fold-change-axis was arbitrary cut at the same value of the panels B and C.

Moreover, I have analyzed the growth potential of human thyroid carcinoma cell lines and, as shown in Figure 11, FRO cells stable exprimenting Cl 2 gene generated a lower (less than 50%) number of colonies than cells transfected with the backbone vector did .

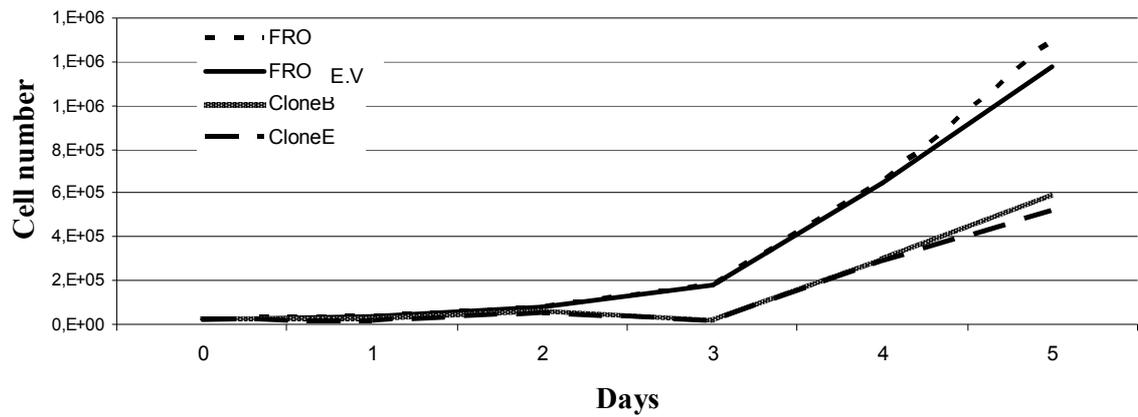


Figure 10. Growth curve of FRO-Cl 2 stable clones (B) and (E), untransfected FRO cells (FRO) and transfected with empty vector (E.V.). After a 5 day observation the number of cells expressing Cl 2 gene was more than 2 times less with respect to control.

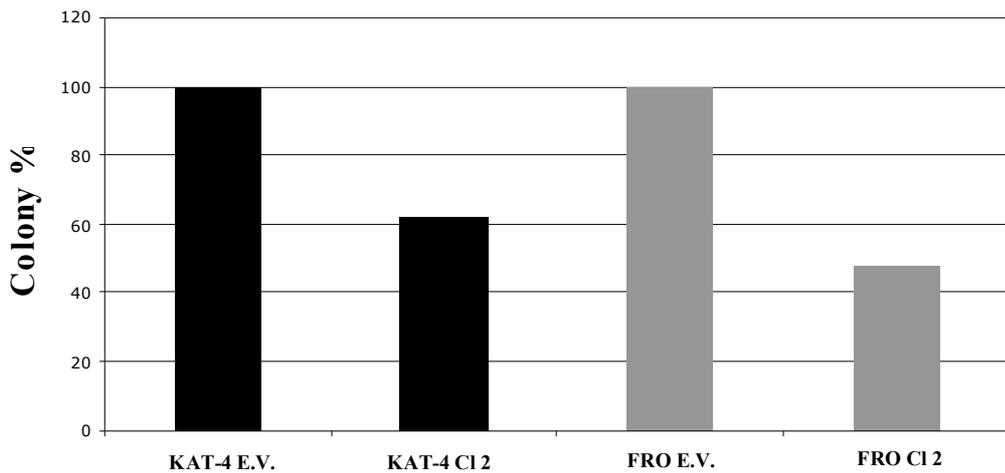


Figure 11. Colony Formation assay. Restoration of Cl 2 gene in two different human thyroid carcinomas cell lines reduces their ability to form colony in culture dish.

4.7

The restoration of the Cl2 expression Cl2 gene inhibits the growth of KAT-4 thyroid carcinoma cell line in semisolid medium.

Consistent results were obtained with a soft agar assay that measured the cell's ability to grow in three-dimensional environment, performed using the KAT-4-Cl 2 cells and KAT-4-E.V. cells. The growth of cell colonies was monitored for three weeks. After just one week, microscope observation revealed the formation of large colonies in plates where the KAT-4 cells transfected with the backbone vector were seeded. Conversely, no colonies could be detected in the plates that contained the KAT-4 cells where Cl 2 gene was restored. After three weeks the colonies of both plates were photographed and counted. As shown in Figure 12, the restoration of the Cl 2 drastically reduces the ability of the FRO cells to grow in agar. Indeed, the number of colonies formed by the KAT-4 cells expressing Cl 2 was much lower in comparison to those generated by the empty vector-transfected KAT-4 cells, and also the size of these colonies was small with the presence of apoptotic-like effect (Figure 12 A).

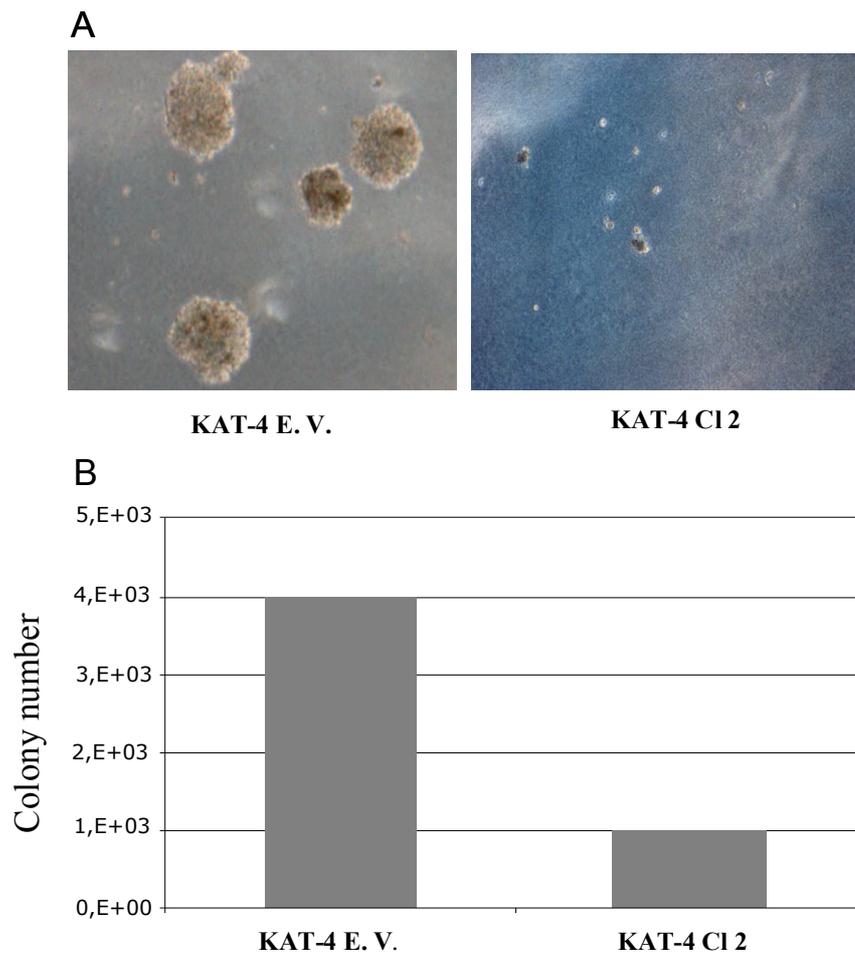


Figure 12. Soft agar assay. Panel A: KAT-4 cells transfected with Cl 2 gene lose their ability to grow in a semi-solid medium (detached from culture disk). Panel B: Histograms represent the number of colonies detected by a microscopy observation.

4.8

Cl 2 affects the cell motility in vitro.

I measured the effect that Cl 2 restoration has on the cell mobility, an important characteristic which marks a malignant phenotype. I performed a migration assay in bi-dimensional environment using the transwell system, and a wound healing assay. As shown in Figure 13 the ability of FRO-E.V. to invade and cross the

polycarbonate Nucleopore filters was higher respect to FRO-C1 2 cells where the expression was re-established. To quantify the invasive capacity of both cells systems I measured by spectrophotometer the amount of dye that the cells had retained after the staining with crystal violet (see materials and methods for details). The ability of FRO-C1 2 cells to cross the membrane was 60% lower with respect to FRO-E.V. cells. Consistent results were also obtained with wound healing assay. FRO-E.V. cells have a greater ability to migrate and to fill the gap generated in the cells monolayer. This is due to the high malignant phenotype that characterized this kind of cell lines . As shown in Figure 14, the cells where C1 2 gene was re-expressed, the capability to move is much lower, in fact the FRO-C1 2 cells fill the gap just as result of cellular division and not as cellular movement. Another important evidence that has been possible to verify looking at the FRO-C1 2 cells was the shape. While the FRO-E.V. or FRO parental cells have a mesenchymal-like shape (Figure 14 B and D), the FRO-C1 2 show a baso-apical polarity that is typical of epithelial cells (Figure 14 A and C). This change in the phenotype may have a crucial role in the migration and in the metastatic process.

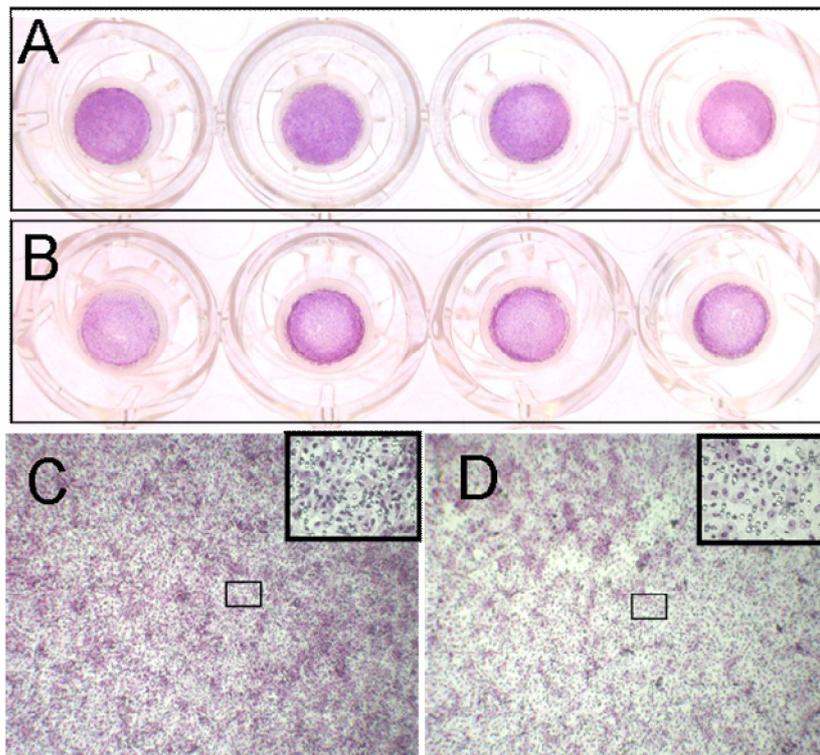


Figure 13. Invasion Assay. Transwells in panel A contain FRO-E V cells, whereas transwells in panel B contain FRO-C1 2 cells. As demonstrated by the weaker staining of transwells in lane B, FRO-C1 2 cells have a reduced ability to invade the membrane of transwells. C and D are optical microscopy pictures of the transwells containing FRO-E V cells and FRO-C1 2 cells respectively.

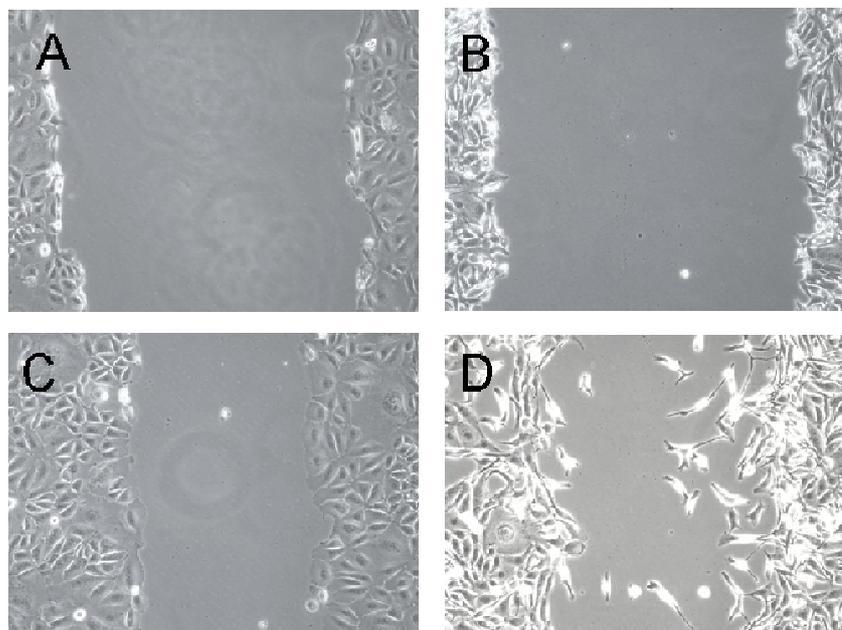


Figure 14 In vitro wound healing assay. Pictures A and C : FRO-C1 2 cells. Pictures B and D: FRO-E V cells. Cells in A and B were photographed immediately after scratch. Cells in C and D were photographed after 20 hours. FRO-E V cells continue to maintain a higher mobility and therefore a more aggressive phenotype.

4.9

Restoration of Cl 2 expression causes cell death.

To understand the role of Cl 2 in cell growth control, I investigated the cell cycle phase distribution of FRO cells transiently transfected with Cl 2 gene through flow cytometric analysis.

As shown in Figure 15, flow cytometric analysis revealed a significant shift of the DNA profile to a sub G1 position. FRO cells expressing Cl 2 present a major percentage of counts in the sub G1 position as compared to FRO-E.V. cells, 27% and 6% respectively. Consistently, a TUNEL assay showed positive staining (red) for the Cl 2 transiently transfected FRO cells, but not for the cells transfected with empty vector (Figure 16). Therefore, these results not only support a role for the Cl 2 gene in the inhibition of cell invasiveness, but also seem to confirm its role in the process of apoptosis.

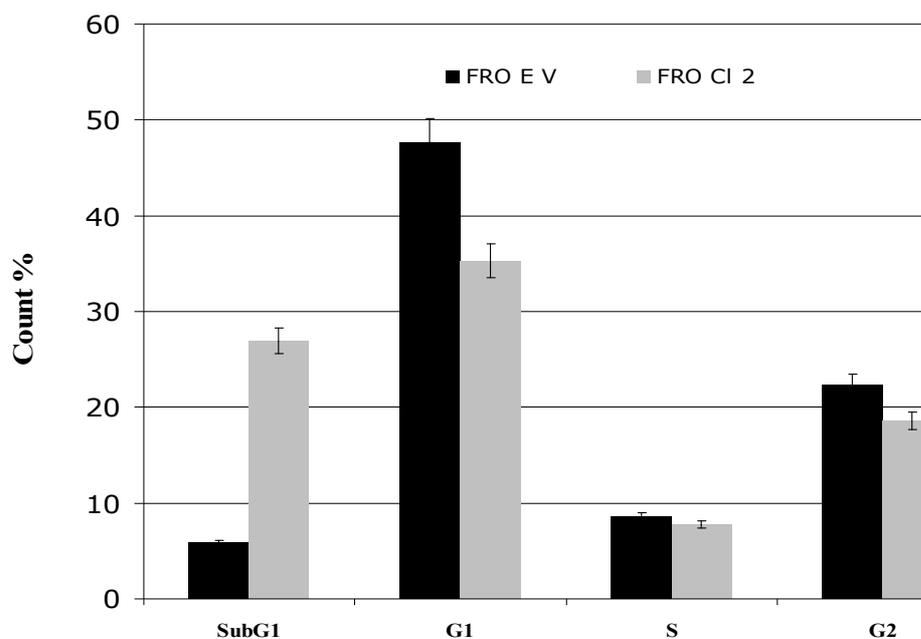


Figure 15. Cell Cycle analysis of FRO cell lines transiently transfected with pCMV.10 vector (FRO-E V) and with pCMV.10-Cl 2 (FRO Cl 2). Re-expression of Cl 2 causes an increment of cells in sub G1 phase.

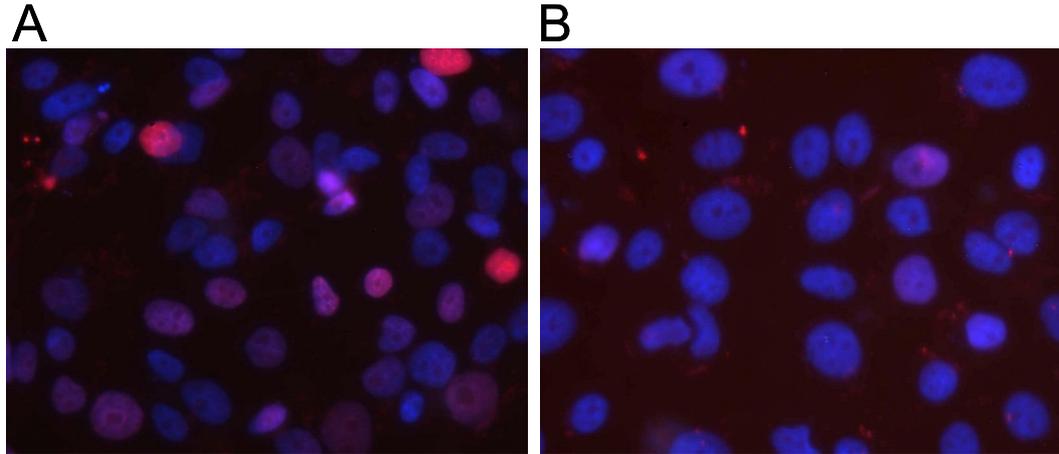


Fig 16. TUNEL assay on FRO cell lines transiently transfected with pCMV10-C1 2 (A) and with empty vector (B). Virtual pictures of apoptotic nucleus were obtained by merging DAPI stained nucleus (blue) with red fluorescence of DNA strand breaks labelled with tetra-methyl-rhodamine-dUPT.

Finally, to investigate the role of C1 2 in tumorigenicity I evaluated the effect of C1 2 protein on the malignant transformed phenotype by analyzing the anchorage-independent growth in athymic mice.

I injected 1×10^7 FRO-C1 2 cells in athymic mice: no development of xenograft tumors was observed even after 6 weeks, whereas they appeared after 3-4 weeks when the same number of untransfected FRO cells was injected. The same result was observed when the same experiments were performed using the KAT-4 cell line.

These results clearly indicate a role of the C1 2 gene in the process of apoptosis and then, in the progression of thyroid carcinogenesis, of the FRO cells where the expression of the C1 2 gene was restored.

4.10

Generation and characterization of Cl 2 null mice

We used gene targeting techniques in embryonic stem (ES) cells to generate a null mutation at the murine Cl 2 genomic locus. We deleted exon I, intron1 and exon 2 which contain the start site of murine Cl 2 gene and replaced them with a neomycin-resistant gene. Both heterozygous and homozygous mutant mice were viable and fertile. PCR and RT-PCR analysis, shown in Figure 16, confirmed the lack of expression of Cl 2 in null mice.

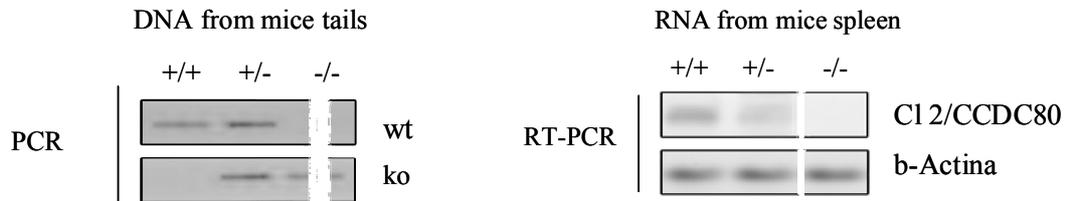


Figure 16. Cl 2 knock-out mice screening. The genotyping of Knock-out Cl 2 mice was performed using PCR and RT-PCR techniques.

Clinical and molecular analysis by Cl 2^{-/-} and Cl 2^{+/-} mice seem to confirm the tumor suppressor role of the Cl 2 gene in thyroid neoplasias. In fact, four out of 16 mice analyzed developed thyroid adenomas and two mice showed ovary carcinomas (Figure 18).

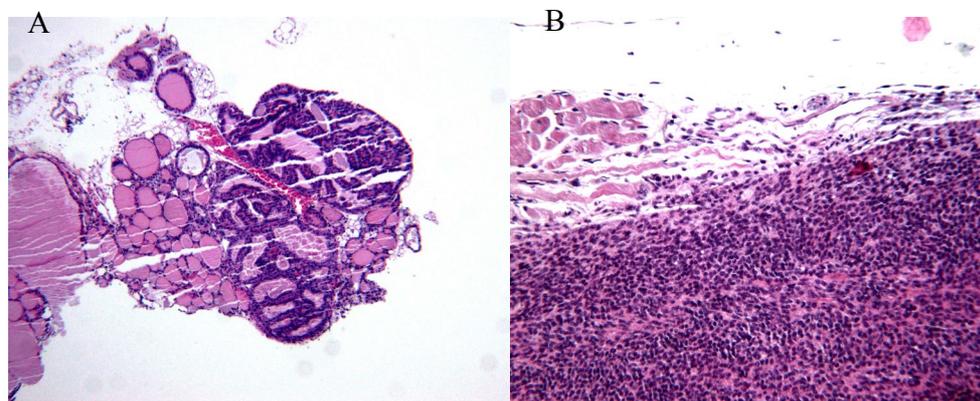


Figure 18. Representative neoplastic lesions in Cl 2 ko mice. A thyroid adenoma. B ovary carcinomas.

Furthermore, I have also analyzed the growth rate of mouse embryonic fibroblasts (MEF) carrying one and two impaired Cl 2 alleles. As shown in Figure 19, MEFs null for Cl 2 have a higher proliferation rate than the wild type MEFs, whereas the Cl 2^{+/-} MEFs show an intermediate behavior suggesting a negative role of the Cl 2 protein in the growth of these cells. Equally, MEFs null for Cl 2 showed a much lower rate of apoptosis in comparison with the wild type and Cl 2^{+/-} MEFs (Figure 20).

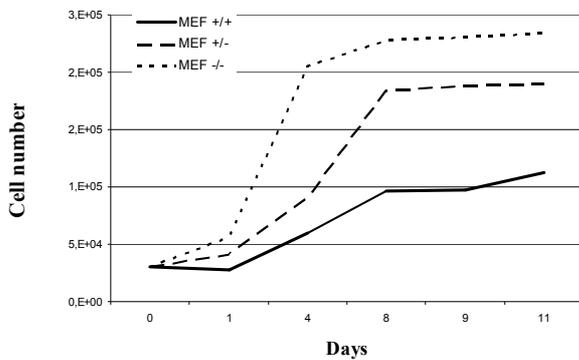


Figure 19. MEFs growth curve. Knock-out MEFs for Cl 2 gene (-/-) grow faster respect WT (+/+). Heterozygous phenotype (+/-) shows an intermediate behavior.

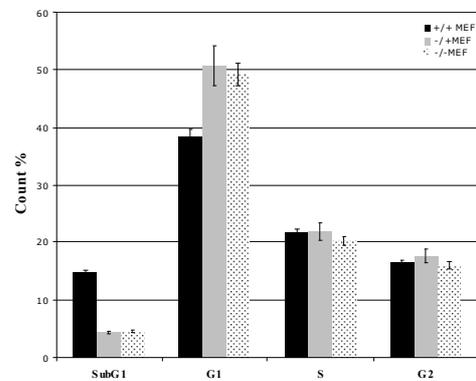


Figure 20. Cell Cycle analysis on MFSs coming from WT mice (+/+), Cl 2^{+/-} and Cl 2^{-/-}. WT MEFs showed a subG1 phase (apoptotic nucleus) 10% higher with respect +/- and -/- MEFs, whereas the G1 phase was 10% less with respect to other genotypes.

Finally, to verify *in vivo* the hypothesis of a tumor suppressor function for Cl 2 I crossed Cl 2^{-/-}, Cl 2^{+/-} and WT mice with RET/PTC1 knock-in mice which developed thyroid tumor easily (Santoro M, 1996). The results confirm that the action of Cl 2 is crucial for thyroid cancerogenesis. Indeed, as shown in Figure 21, the thyroid tumor of Cl 2^{-/-}_RET/PTC1 mice was enormous when compared with thyroid of RET/PTC1 transgenic mice. Moreover, as reported in Table 5

these types of tumors not only have big dimension but also present histopathological characteristics more aggressive with infiltrating and solid pattern that are rarely found in classical PTC mice.

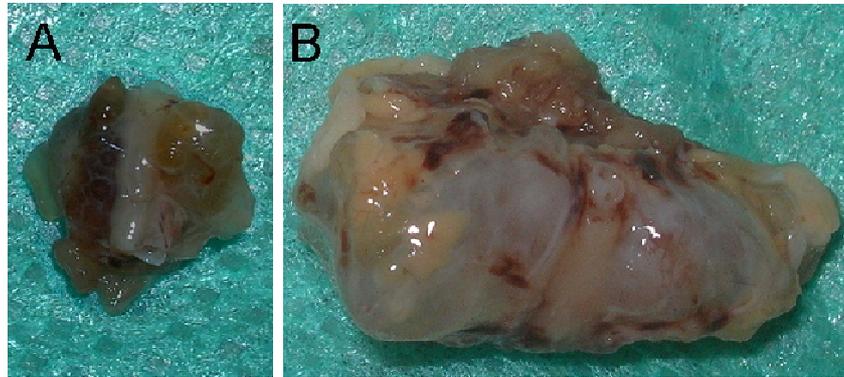


Figure 21. Mouse thyroid tumors. A: Representative thyroid tumor from an RET/PTC1 knock-in mouse. B: Representative thyroid tumor developed from a considerable number of mice originated by the cross of RET/PTC1 knock-in mouse with Cl 2 knock-out mouse.

Genotype	RET/PTC1 Cl 2 +/+	RET/PTC1 Cl 2 +/-	RET/PTC1 Cl 2 -/-
Total Mice	5	10	8
<u>Pathological lesions</u>			
Thyroid Carcinoma	3	5	7
Iperplasia	1	0	1
Normal thyroid	1	5	0
<u>Diagnosis</u>			
PTC	3	2	1
PTC solid pattern	0	3	3
PTC infiltrating	0	0	3
<u>Dimension range</u>			
1-2 mm	1	4	2
2-5 mm	4	4	4
5-10 mm	0	2	2
<u>Sex</u>			
Female	2	6	3
Male	3	4	5

Table 5. Summary of histopathological characteristics developed by RET/PTC1-Cl 2 mice.

DISCUSSION

Thyroid carcinoma is a common malignant tumor with a good prognosis if timely diagnosed. The enlargement number of emerging candidate diagnostic thyroid cancer markers can improve the clinical treatment of thyroid cancer. To day, several oncogenes have been demonstrated to be involved in human thyroid carcinomas, particularly in papillary histotype, but little is known with regard to tumor suppressor genes. In addition, about 20-25% of carcinomas affecting the thyroid gland seem not to present mutations or expression changes of well-known oncogenes. Therefore, other mechanisms such as up- or down-regulation of transcription level of some genes may have an important role in thyroid carcinogenesis.

Recently (Visconti R, 2003) was reported the identification of a gene, named Cl 2, and subsequently CCDC80, downregulated in a panel of few cases of thyroid carcinomas, even though its expression was enormously upregulated in rat thyroid cells infected with the E1A gene of Adenovirus. To explain this contrasting behavior of Cl 2 expression in PC E1A cell lines and thyroid carcinoma, it was hypothesized that abundant Cl 2 mRNA level and consequently elevated levels of protein could contribute to the high apoptotic rate of PC E1A cells, suggesting a tumor suppressor role for Cl2 gene.

The results reported in the present study seem to support a role of Cl 2 gene in

human thyroid carcinogenesis through its involvement in the control of cell growth, invasion and apoptosis. Indeed, I confirmed, by qRT-PCR, the decrease in Cl 2 expression in thyroid carcinoma analyzing a large number of cases. A severe reduction of Cl 2 mRNA was observed in the follicular variant of papillary carcinomas, in which the average down-regulation value was 60 fold. Moreover, in some samples of this histotype Cl 2 specific mRNA was almost undetectable. A drastic reduction of Cl 2 was also present in the classic form of papillary carcinoma, in follicular and anaplastic carcinomas with an average down-regulation value of 15-20 fold. Interesting results were obtained when Cl 2 protein expression was analyzed by immunohistochemistry. I found that Cl 2 protein in thyroid carcinomas presents not only a downregulation but also a different localization. In fact, in normal thyroid tissue the signal of immunostaining is clearly nuclear but becomes cytoplasmatic in papillary and follicular carcinomas disappearing in almost all anaplastic carcinomas. LOH at the Cl 2 locus and an increase of the methylation status of single CpG close to TSS seem to account for the reduced expression of Cl 2. Indeed, LOH at Cl 2 locus was observed in 50% of the follicular variants of papillary carcinomas, and was well correlated with the downregulation of Cl 2 gene expression. As far as the methylation status of the Cl 2 promoter is concerned, although a dense CpG island located in the second exon was not differentially methylated, a single CpG site very close to TSS (+21) was hypermethylated in tumors (42,5%) compared

to normal tissues. Even if the region surrounding the TSS is very poor in CpG sites, the differential methylation of a single CpG sites located in a strategic position (+21) may potentially affect the binding of methyl-binding proteins at promoter region and, as a consequence, may have a consistent impact on the transcriptional potential of the gene. Moreover, unexpectedly, and at odds with the recent publication that indicates a downregulation of Cl 2 expression by beta-catenin, no inverse correlation, was found between beta-catenin and Cl 2 expression. I observed that the decrease in Cl 2 expression is more marked where the beta-catenin expression is not increased. Furthermore, the significant Cl 2 downregulation in human breast, colon ,and particularly, ovarian carcinoma propose it as a general event in the process of carcinogenesis. Further evidence supports the relation between Cl 2 gene and carcinogenesis coming from the restoration of Cl 2 expression in human thyroid carcinoma cell lines that cause a drastic reduction of cell growth rate. I also showed an inhibitory effect of Cl 2 overexpression on cell invasion. Moreover, a cytofluorimetric assay demonstrated a shift of the DNA profile to a sub G1 position, that is indicative of apoptosis, when the expression of Cl 2 was restored in thyroid carcinoma cell lines. Then, the ability of Cl 2 to induce apoptosis was confirmed by a TUNEL assay. Furthermore, the cells re-expressing the Cl 2 lost the typical markers of neoplastic transformation since they were not able to grow efficiently in semisolid medium and did not induce tumors when injected into athymic mice.

The generation of mice knock out for the Cl 2 gene seemed to confirm a tumor suppressor role for this gene. Indeed, MEFs Cl 2^{-/-} have a higher proliferation rate with respect to the wild type, whereas the MEFs^{+/-} show an intermediate behaviour. Moreover, the MEFs KO for Cl 2 were less susceptible to apoptosis in comparison to the MEFs^{+/-} and ^{+/+}. In addition, these mice developed thyroid adenomas with a significant frequency, an event occurring very rarely in normal wild type (Biancifiori C, 1979), and three ovarian carcinomas. This last result is also consistent with the drastic loss of Cl 2 expression in human ovarian carcinomas. Finally, I demonstrated through mouse models, that the loss of Cl 2 gene is crucial for thyroid cancerogenesis. In fact, crossing RET/PTC1 knock-in mice with Cl 2 knock-out mice I observed a synergy between oncogenetic ability of RET/PTC1 oncogene and loss of oncosuppressor capacity of Cl 2. The thyroid gland of mice resulting by the cross showed biggest dimensions due to neoplastic transformation with very relevant histopathological lesions. In conclusion, all the data reported here indicate a critical role of the loss of CL 2 expression in the process of thyroid carcinogenesis and likely other tissues.

ACKNOWLEDGEMENTS

I would like to acknowledge Prof. Francesco Salvatore who directed this Doctorate Program.

I would like to thank all the members of my laboratory for the support, the friendship and the enlightening scientific discussions. I am very grateful to Dr. Enza Leone and Dr Eleonora Borbone who helped me with some experimental assays.

I wish to thank Dr. Gennaro Chiappetta and his group at the Istituto Nazionale dei Tumori, Fondazione Pascale, Naples.

I also wish to thank all the friends and colleagues at the CEINGE Biotecnologie Avanzate which made these years a great experience.

A special thank goes to prof. Alfredo Fusco, who supervised my work. His extraordinary knowledge of oncology, his great enthusiasm and kindness are really unique. It has been a pleasure for me to work in his laboratory at Naples Oncogenomic Center (NOGEC) in the CEINGE institute.

Bibliography

- 1) Aoki K, Sun YJ, Aoki S, Wada K, Wada E. Cloning, expression, and mapping of a gene that is upregulated in adipose tissue of mice deficient in bombesin receptor subtype-3. *Biochem Biophys Res Commun.* 2002 Feb 1;290(4):1282-8
- 2) Biancifiori, C. Pathology of tumors in laboratory animals. In: V. S. Turonsov (ed.), *Tumors of the Mouse (IARC Scientific Pub. No. 23), Vol. II*, pp. 451-163. Lyon, France: International Agency for Research on Cancer, 1979.
- 3) Bommer GT, Jäger C, Dürr EM, Baehs S, Eichhorst ST, Brabletz T, Hu G, Fröhlich T, Arnold G, Kress DC, Göke B, Fearon ER, Kolligs FT. DRO1, a gene down-regulated by oncogenes, mediates growth inhibition in colon and pancreatic cancer cells. *J Biol Chem.* 2005 Mar 4;280(9):7962-75. Epub 2004 Nov 24
- 4) Bruni P, Boccia A, Baldassarre G, Trapasso F, Santoro M, Chiappetta G, Fusco A, Viglietto G. PTEN expression is reduced in a subset of sporadic thyroid carcinomas: evidence that PTEN-growth suppressing activity in thyroid cancer cells mediated by p27kip1. *Oncogene.* 2000 Jun 29;19(28):3146-55. PubMed PMID: 10918569
- 5) Chinnadurai G. Adenovirus E1a as a tumor-suppressor gene. *Oncogene.* 1992Jul;7(7):1255-8. Review
- 6) Fagin JA, Matsuo K, Karmakar A, Chen DL, Tang SH, Koeffler HP. High prevalence of mutations of p53 gene in poorly differentiated human thyroid carcinomas. *J Clin Invest* 1993; 91:179–84.
- 7) Fusco A, Viglietto G, Santoro M. A new mechanism of BRAF activation in human thyroid papillary carcinomas. *J Clin Invest.* 2005 Jan; 115(1): 94-101.

- 8) Hay ID. Papillary thyroid carcinoma. *Endocrinol Metab Clin North Am.* 1990;10:545–576.
- 9) Hedinger C, Williams ED, Sobin LH. The WHO histological classification of thyroid tumors: a commentary on the second edition. *Cancer.* 1989 Mar 1;63(5):908-11.
- 10) Iuliano R, Trapasso F, Le Pera I, et al. An adenovirus carrying the rat protein tyrosine phosphatase eta suppresses the growth of human thyroid carcinoma cell lines *in vitro* and *in vivo*. *Cancer Res* 2003;63:882–6
- 11) Kazakov VS, Demidchik EP, Astakhova LN. Thyroid cancer after Chernobyl. *Nature.* 1992 Sep 3;359(6390):21.
- 12) Kenneth J. Livak and Thomas D. Schmittgen. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. 2001 *Methods* 25, 401-408.
- 13) Knudson AG Jr: Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci U S A* 68:820-823, 1971
- 14) Kondo T, Ezzat S, Asa SL. Pathogenetic mechanisms in thyroid follicular-cell neoplasia. *Nat Rev Cancer.* 2006 Apr;6(4):292-306. Review.
- 15) Kroll TG, Sarraf P, Pecciarini L, et al. PAX-8-PPAR $\{\gamma\}$ 1 fusion oncogene in human thyroid carcinoma. *Science* 2000; 289:1357–60.
- 16) Kurosawa H, Goi K, Inukai T, Inaba T, Chang KS, Shinjyo T, Rakestraw KM, Naeve CW, Look AT. Two candidate downstream target genes for E2A-HLF. *Blood.* 1999 Jan 1;93(1):321-32.
- 17) Li Volsi VA, Asa SL. The demise of follicular carcinoma of the thyroid gland. *Thyroid.* 1994;4:233–236
- 18) Liu Y, Monticone M, Tonachini L, Mastrogiacomo M, Marigo V, Cancedda R, Castagnola P. URB expression in human bone marrow stromal cells and during mouse development. *Biochem Biophys Res Commun.* 2004 Sep 17;322(2):497-507

- 19) Lupas AN and Gruber M The structure of alpha-helical coiled coils. *Advances in Protein Chemistry* 70: 37-78 (2005)
- 20) Marcantonio D, Chalifour LE, Alaoui-Jamali MA, Alpert L, Huynh HT. Cloning and characterization of a novel gene that is regulated by estrogen and is associated with mammary gland carcinogenesis. *Endocrinology*. 2001 Jun;142(6):2409-18.
- 21) Montagnier L, Macpherson I. Selective development in agar of hamster cells transformed by the polyoma virus. *Virology*. 1964 Jun;23:291-4
- 22) Mu H, Ohta K, Kuriyama S, Shimada N, Tanihara H, Yasuda K, Tanaka H. Equarin, a novel soluble molecule expressed with polarity at chick embryonic lens equator, is involved in eye formation. *Mech Dev*. 2003 Feb;120(2):143-55.
- 23) Nikiforov Y, Gnepp DR. Pediatric thyroid cancer after the Chernobyl disaster. Pathomorphologic study of 84 cases (1991-1992) from the Republic of Belarus. *Cancer*. 1994 Jul 15;74(2):748-66.
- 24) Nikiforova MN, Lynch RA, Biddinger PW, Alexander EK, Dorn GW 2nd, Tallini G, Kroll TG, Nikiforov YE. RAS point mutations and PAX8-PPAR gamma rearrangement in thyroid tumors: evidence for distinct molecular pathways in thyroid follicular carcinoma. *J Clin Endocrinol Metab*. 2003 May;88(5):2318-26.
- 25) Okada T, Nishizawa H, Kurata A, Tamba S, Sonoda M, Yasui A, Kuroda Y, Hibuse T, Maeda N, Kihara S, Hadama T, Tobita K, Akamatsu S, Maeda K, Shimomura I, Funahashi T. URB is abundantly expressed in adipose tissue and dysregulated in obesity. *Biochem Biophys Res Commun*. 2008 Mar 7;367(2):370-6. Epub 2008 Jan 4.
- 26) Paige AJ: Redefining tumor suppressor genes: exceptions to the two-hit hypothesis. *Cell Mol Life Sci* 60:2147-2163, 2003.

- 27) Pan J, Nakanishi K, Yutsudo M, Inoue H, Li Q, Oka K, Yoshioka N, Hakura A. Isolation of a novel gene down-regulated by v-src. *FEBS Lett.* 1996 Mar 25;383(1-2):21-5.
- 28) Pierotti MA, Greco A. Oncogenic rearrangements of the NTRK1/NGF receptor. *Cancer Lett* 2006;232:90–8.
- 29) Portella G, Ferulano G, Santoro M, Grieco M, Fusco A, Vecchio G. The Kirsten murine sarcoma virus induces rat thyroid carcinomas in vivo. *Oncogene.* 1989 Feb;4(2):181-8.
- 30) Ron E, Lubin JH, Shore RE, Mabuchi K, Modan B, Pottern LM, Schneider AB, Tucker MA, Boice JD Jr. Thyroid cancer after exposure to external radiation: a pooled analysis of seven studies. *Radiat Res.* 1995 Mar;141(3):259-77. PubMed PMID: 7871153.
- 31) Salabè GB. Aetiology of thyroid cancer: an epidemiological overview. *Thyroidology.* 1994 Apr;6(1):11-9. Review.
- 32) Saltman B, Singh B, Hedvat CV, Wreesmann VB, Ghossein R. Patterns of expression of cell cycle/apoptosis genes along the spectrum of thyroid carcinoma progression. *Surgery.* 2006 Dec;140(6):899-905; discussion 905-6.
- 33) Santoro M, Chiappetta G, Cerrato A, Salvatore D, Zhang L, Manzo G, Picone A, Portella G, Santelli G, Vecchio G, Fusco A. Development of thyroid papillary carcinomas secondary to tissue-specific expression of the RET/PTC1 oncogene in transgenic mice. *Oncogene.* 1996 Apr 18;12(8):1821-6.
- 34) Suarez HG, du Villard JA, Severino M, et al. Presence of mutations in all three ras genes in human thyroid tumors. *Oncogene* 1990; 5:565–70.
- 35) Tremblay F, Revett T, Huard C, Zhang Y, Tobin JF, Martinez RV, Gimeno RE. Bidirectional modulation of adipogenesis by the secreted protein Ccdc80/DRO1/URB. *J Biol Chem.* 2009 Mar 20;284(12):8136-47. Epub

2009 Jan 13.

- 36) van der Laan BF, Baris G, Gregor RT, Hilgers FJ, Balm AJ. Radiation-induced tumours of the head and neck. *J Laryngol Otol.* 1995 Apr;109(4):346-9.
- 37) Visconti R, Schepis F, Iuliano R, Pierantoni GM, Zhang L, Carlomagno F, Battaglia C, Martelli ML, Trapasso F, Santoro M, Fusco A. Cloning and molecular characterization of a novel gene strongly induced by the adenovirus E1A gene in rat thyroid cells. *Oncogene.* 2003 Feb 20;22(7):1087-97
- 38) White E. (1995). *Current Topics in Microbiology and Immunology: The Molecular Repertoire of Adenovirus.* Doerfler W (ed). Springer-Verlag: Berlin, Heidelberg, pp. 33–58.
- 39) Whyte P, Ruley HE, Harlow E. Two regions of the adenovirus early region 1A proteins are required for transformation. *J Virol.* 1988 Jan;62(1):257-65.