ANTICANCER RESEARCH *24*: 1495-1500 (2004)

Nuclear Methylation Levels in Normal and Cancerous Thyroid Cells

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Abstract. *Background: Most cancers show abnormal DNA methylation and a positive correlation between hypomethylation and tumour progression. Patients and Methods: In our laboratory the extent of DNA methylation in individual nuclei in normal, cancer and non-cancer thyroid tissue samples was quantified according to a previously described method of computer-assisted semi-quantitative analysis. Cancer and non-cancer samples were obtained from nine patients with different thyroid pathologies (one multinodular goitre and eight carcinomas). Quantitative analysis was performed in two sets of samples, i.e. individual nuclei from touch preparations and from tissue sections. Results: In all cancer specimens a statistically significant decrease of heterochromatin methylation was consistently observed. In both sets of samples a direct correlation was consistently observed between the extent of chromatin demethylation and the degree of malignancy. Conclusion: Our preliminary results suggest that our method of cell-by-cell detection of intranuclear methylation abnormalities may be a useful tool in early identification of thyroid cancer lesions.*

Mammalian cells modify their DNA by means of a post-replicative process consisting of adding a methyl group to the 5' position of the cytosine ring in the two-base sequence 5'-CpG-3'. The location of 5-methylcytosine in the constitutive heterochromatin of mammalian cells was first described by Miller *et al.* (1). DNA methylation plays a role in the control of gene expression: expressed genes are hypomethylated while inactivated genes are generally highly methylated (2, 3).

Aberrant DNA methylation has been detected in most human and mammalian cancers: the transformed cells often show increased methyltransferase levels and abnormal methylation of the CpG islands in the promoter regions of several genes (4, 5). Recently, Costello *et al.* (6) surveyed the methylation status of 1184 CpG islands in 98 human tumours, and estimated that an average of 600 CpG islands were abnormally methylated. Promoter hypermethylation is directly related to the transcriptional inactivation of cancer suppressor genes, or of genes involved in DNA repair, DNA differentiation and in the cell cycle control (7).

However, the total amount of 5-methylcytosine in the genomic DNA of tumour cells is generally lower than in their normal counterparts. Many human cancers show a positive correlation between chromatin hypomethylation and tumour progression. The mechanisms by which the loss of genomic methylation may contribute to malignant transformation are still unclear. Oncogene activation has been suggested to be a mechanism providing selective advantages to transformed cells (8). It has also been proposed that the hypomethylation of non-promoter regions may contribute to genomic and chromosomal instability (9), activation of endogenous parasitic sequences (10) and loss of imprinting. A study of 136 breast cancer lesions showed a direct correlation between the extent of genomic hypomethylation, tumour size, disease stage and degree of malignancy (11). On these grounds it was recently suggested that DNA hypomethylation could be considered as a biological marker having a prognostic value (12).

Over the last few years we have developed a computer-assisted method for the quantitative analysis of chromatin methylation by indirect immunolabelling with anti-5-MeC monoclonal antibodies (13). We present the preliminary results obtained by analysing the methylation status in different types of malignant thyroid tumours as compared to normal tissue.

Patients and Methods

The present study included nine patients, four men and five women (age range: 33-60 years) subjected to total thyroidectomy: in one case for multinodular goitre and in the remaining eight cases for thyroid carcinoma. According to the recent thyroid tumour

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Key Words: Thyroid cancer, hypomethylation.

Table I. *Patients and histopathological diagnosis of the thyroid tissue samples*

Patients	Age years	Sex	Tissue sample	Histological diagnosis	Tumor size	Stage	Grading
A	56	F	Istmus	Multinodular Goitre			
B	37	F	Left Lobe	Papillary Microcarcinoma	T1	I	G2
C	38	M	Left Lobe	Papillary Carcinoma	T1	I	G1
D	33	M	Left Lobe	Follicular Carcinoma	T2	I	G1
E	42	M	Right Lobe	Papillary Carcinoma Follicular variant	T2	I	G1
F	45	M	Left Lobe	Medullary Carcinoma	T2	II	G2
G	40	F	Left Lobe	Papillary Carcinoma	T1	I	G2
H	33	F	Right Lobe	Medullary Carcinoma Small cell variant	T1	I	G1
I	60	I	Left Lobe	Follicular Carcinoma	T3	II	G2

The histologically normal tissue was used as control.

classification (14), these eight cases were pathologically classified as papillary microcarcinoma (one case), papillary carcinoma (three cases), follicular carcinoma (two cases) or medullary carcinoma (two cases). The stage of the tumour was determined using the current criteria of the UICC TNM classification (1997). In all patients the thyroid tissue, which was classified histologically as normal, was used as control (Table I).

Analysis of indirect immunolabelling in individual nuclei. Touch preparations of isolated cells were obtained from resected surgical samples. In order to quantify the methylation levels in individual nuclei, a complete set of touch preparations was analysed for each patient. The slides were fixed in cold methanol-acetic acid (3:1) for 15 minutes and then submitted to UV irradiation to denature the DNA. Indirect immunolabelling was performed by anti 5-MeC monoclonal antibodies, previously produced and characterised (15). The second goat-anti-mouse antibodies conjugated to peroxidase were from Sigma (Milan, Italy). The binding of antibodies was detected by 4-alphachloronaphtol (4-ClN) as the substrate for peroxidase in the presence of hydrogen peroxide.

Immunolabelling of tissue sections. Samples obtained from normal, multinodular goitre and malignant thyroid tissue were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections (4-μm thick) were cut for histological examination (haematoxylin-eosin staining) and for immunohistochemical staining. Sections for immunostaining were de-waxed with xylene and treated with 2% H_2O_2 for 10 minutes to block endogenous peroxidases. The slides were placed in citrate buffer at pH 6.0 in a microwave oven at full potency for 3 cycles (5, 5 and 3 minutes). The slides were then immersed in 3.5 HCl for 15 minutes at room temperature in order to expose the methylated sites. All unspecific binding sites were blocked with a non-fat milk (5%) solution in PBS buffer and Tween (0.1%) for 15 minutes.

Sections were incubated with the anti-5-MeC monoclonal antibodies at a 5 μg/ml concentration for 1 hour at 37˚C, followed by incubation at 37˚C with a second goat-anti-mouse antibody conjugated to peroxidase (Sigma, Milan, Italy). Diaminobenzidine tetrahydrochloride (DAB) was used as the substrate to visualise the antigen-antibody complex. In all cases the cells to be analysed – well spread and not overlapping – were selected by three experienced independent observers. Digital images were acquired with a Leica Diaplan microscope (lens mag. 63x) equipped with a Leitz camera and analysed by Image Pro-Plus 3.1 software (Media Cybernetics, Milan).

DNA methylation was evaluated by measuring the number, size and optical density (OD) of the labelled heterochromatic regions ('spots') as in our previously reported investigations (13). The DNA methylation index was obtained by multiplying the area and intensity values of the spots. The optical density of the individual cell nuclei was determined in tissue sections.

Table II. *Chromatin methylation in normal and pathological nuclei from touch preparations.*

	Normal tissue	Multi-nodular goitre	Papillary micro-carcinoma	Papillary carcinoma	Follicular carcinoma	Papillary carcinoma (follicular variant)	Medullary carcinoma
No. of nuclei	70	66	72	69	72	54	70
Mean area spots (μm^2)	4.36 ± 0.39	3.38 ±0.28*	2.34 ± 0.21***	0.99 ± 0.08***	0.49 ± 0.10***	0.25 ± 0.07***	0.009 ± 0.006***
Mean OD spots	2.09 ± 0.202	1.73 ± 0.09 **	0.97 ± 0.08 ***	0.55 ± 0.05***	0.39 ± 0.06***	0.33 ± 0.09***	0.04 ± 0.02***
Mean no. of spots/cell	7.03	5.62	2.94	1.83	1.30	1.15	0.13
DNA methylation index	1102.7	884.7	532.1	181.4	74.5	33.7	0.2

Spots: heterochromatic antibody-positive regions
OD: optical density
Data expressed as means ± S.E.
***: $p<0.001$; **: $p<0.01$; *: $p<0.05$ (Student's *t*-test for unpaired data)

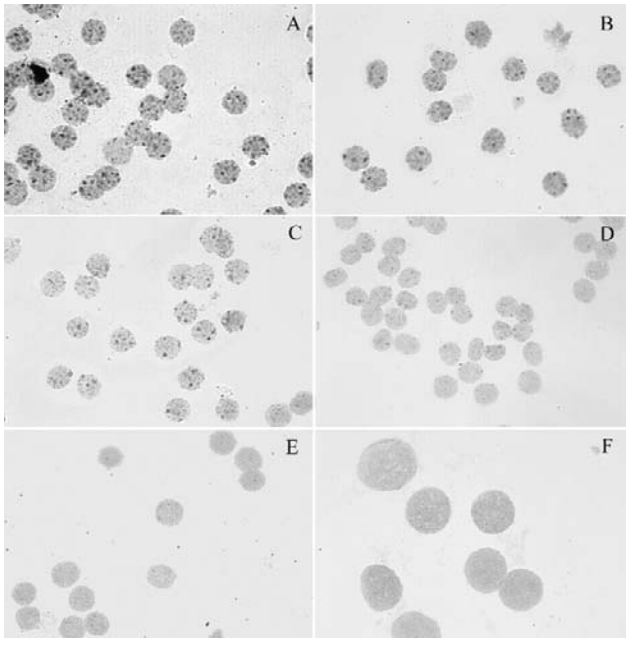

Figure 1. *Digital images of individual nuclei from normal and pathological cells. Indirect immunolabelling by anti-5 MeC antibodies. Substrate 4ClN. A: normal cells; B: papillary microcarcinoma; C: papillary carcinoma; D: follicular carcinoma; E: papillary carcinoma, follicular variant; F: medullary carcinoma.*

The statistical significance of the results was evaluated by the two-tailed Student's *t*-test for unpaired data.

Results

Cytological samples of individual nuclei obtained by touch preparations and a set of individual nuclei within tissue sections of thyroid samples from each non-cancer and cancer resected lobe were analysed (Table I).

The cell-by-cell analysis of normal and pathological individual nuclei showed that there were statistically significant differences in cells obtained from samples with different pathological characteristics and/or levels of malignancy. The areas and OD of the heterochromatic regions ("spots"), as well as the average number of spots per cell decreased as the malignancy levels of cancer cells increased. The methylation index, representing the amount of bound antibodies to the cell nucleus decreased accordingly (Table II, Figures 1 and 2). Also, the extent of nuclear methylation of individual nuclei was analysed in thyroid tissue sections from control and pathological samples taken from the same subjects. The OD of the nuclei in non-cancer tissues was consistently and significantly higher than the OD of nuclei from cancer tissues (Table III).

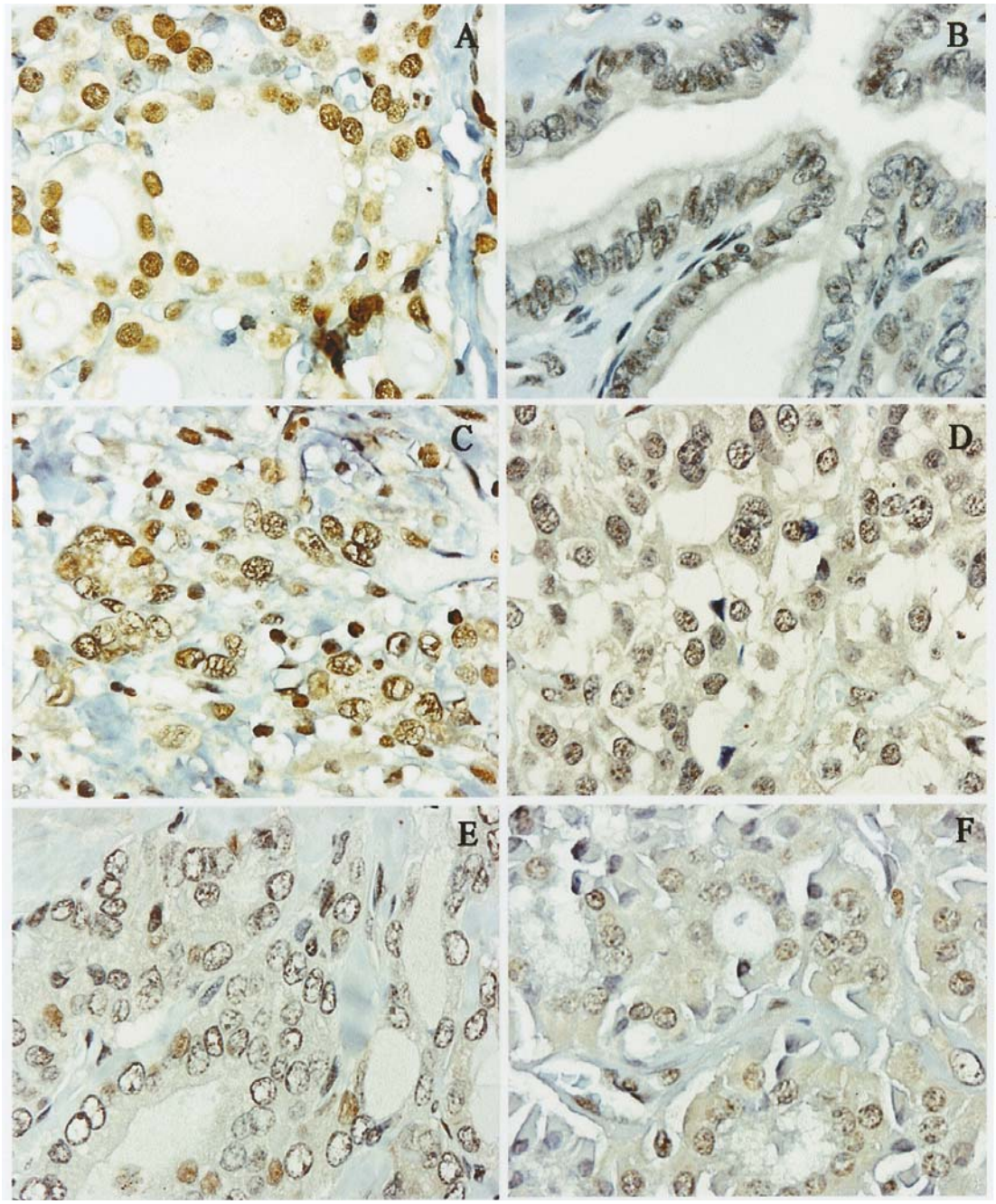

Figure 2. *Digital images of tissue sections. Indirect immunolabelling by anti-5-MeC antibodies. Substrate DAB; counterstaining: Mayer's hematoxylin. A: normal cells; B: papillary microcarcinoma; C: papillary carcinoma; D: follicular carcinoma; E: papillary carcinoma, follicular variant; F: medullary carcinoma.*

This choice enabled us to compare the methylation levels of individual nuclei in the two sets of specimens and demonstrated that the methylation level is significantly lower in cancer cells than it is in the control samples (Figure 3 and Table III).

Discussion

Alterations in global DNA methylation levels have been observed in many human cancers, but whether such alterations are relevant in tumour initiation and progression is unknown.

Table III. *Analysis of anti-5-MeC antibody positive nuclei in thyroid tissue sections.*

	Normal tissue	Multi-nodular goitre	Papillary micro-carcinoma	Papillary carcinoma	Follicular carcinoma	Papillary carcinoma (follicular variant)	Medullary carcinoma
No. of nuclei	84	80	84	69	84	42	84
Mean OD nuclei	0.171±0.002	0.148±0.007*	0.140±0.003**	0.143±0.004**	0.099±0.002**	0.107±0.001**	0.123±0.002**

OD: optical density
Data expressed as means ± S.E.
**: $p<0.001$; *: $p<0.01$ (Student's *t*-test for unpaired data)

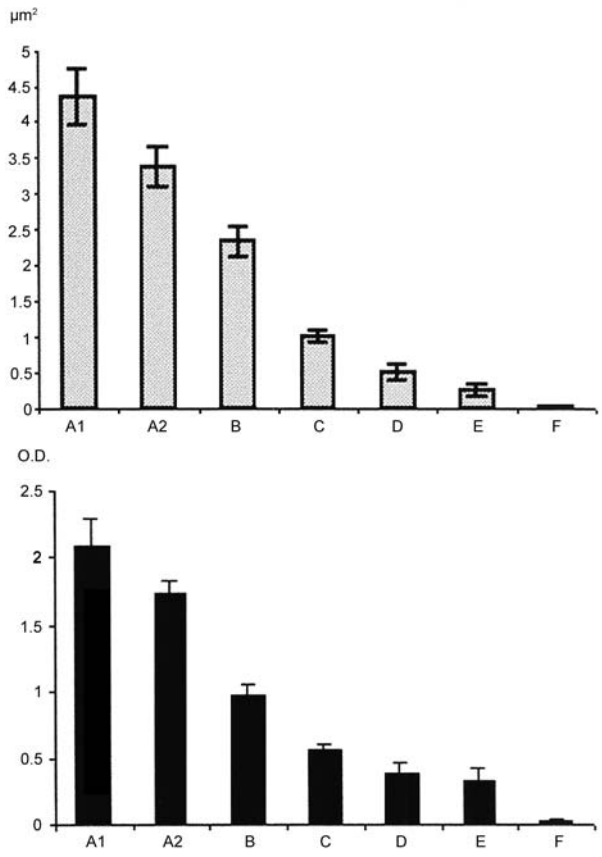

Figure 3. *Mean area (µm²) and optical density (OD) of the heterochromatic regions in individual nuclei from normal and pathological cells.*

It has been suggested that cellular hypomethylation may contribute to carcinogenesis, genomic instability and activation of oncogenes (7-9). Several authors (16, 17) report that DNA hypomethylation increases the progresssion of squamous lung cancer and that DNA hypomethylation increases with tumour progression. It has been suggested that the maintenance of normal DNA methylation may impede the multistep sequence: bronchial epithelial hyperplasia, dysplasia, cancer *in situ*, invasive squamous cell cancer. Normal DNA methylation in hyperplastic or dysplastic bronchial lesions may prevent the malignant transformation process. By contrast, DNA hypomethylation occurring in these same lesions may indicate a risk for the development of invasive cancer. These results were obtained through the quantitative analysis of nuclear methylation in both individual nuclei and in nuclei from histological tissue sections of surgical specimens. We recently demonstrated a progressive chromatin demethylation in precancerous and cancer cells from uterine cervix samples (18), by analysis of DNA methylation in individual cells from cytofuge samples and measurement of methylation levels in tissue sections.

The results of this study show that DNA hypomethylation is significantly evident in thyroid cancer as well as in other cancers (breast, uterine cervix, stomach and colon-rectum) (11, 18-20). We also observed a significant relationship between the levels of nuclear chromatin demethylation and the biological malignancy of the different histological types of thyroid cancer. Indeed, the most aggressive tumours (medullary carcinoma) had very high demethylation levels, while the less aggressive tumours had demethylation levels more similar to those in the hyperplastic tissue of multinodular goitre; and moderately aggressive tumours had intermediate demethylation levels. Therefore, the methylation levels observed in multinodular goitre hyperplastic tissue were slightly lower than in normal control tissue and gradually decreased with neoplastic aggressiveness.

We do not know whether demethylation occurs and, if so, what the levels are in non neoplastic thyroid lesions or in those in which the malignancy or non-malignancy is difficult to estabilish histologically. It would be interesting to study whether nuclear methylation is relevant in differentiating thyroid nodular formations that are difficult to interpret. We know that in many cases the differentiation between nodular hyperplasia and

neoplasia may be impossible and that 0.5-4% of multinodular goitres are associated with malignancy (14, 21). Although needle biopsy is the best method available for diagnosis, false-negative diagnoses do occur particularly when differentiating benign from malignant thyroid nodules. (21-24). It is generally impossible to distinguish follicular adenomas from well-differentiated follicular carcinomas on the basis of cytological or histological examination (14, 23). Also, papillary adenomas and their follicular variants may be misdiagnosed by cytology. Approximately 5% of microfollicular and Hurtle cell tumours show microscopic features indicative of malignancy often only after examining multiple tissue sections (14, 22, 26, 27).

It is not difficult to understand the implications of these diagnostic problems at the prognostic and therapeutic level. Moreover, quantification of the levels of methylation of lesions with uncertain benign or malignant diagnosis may constitute a reliable means of differentiation. Drawing attention to the problem could give rise to new prospects for diagnostic purposes as well as for the surgical treatment of the different types of thyroid cancer.

Acknowledgements

We are grateful to Drs. Antonella di Noia and Veronica Taddeo for their skilful laboratory assistance and to Mr. Domenico Grillo for the quantitative analysis and computer graphics.

This research was partially supported by the Italian Ministry for Scientific Research (Murst) to Claudio Grappelli.

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Received January 20, 2004
Accepted March 18, 2004