SPANDIDOS PUBLICATIONS

Comparative genomic hybridization, *BRAF, RAS, RET,* and oligo-array analysis in aneuploid papillary thyroid carcinomas

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Abstract. Aneuploidy in papillary thyroid carcinomas (PTCs) is considered a marker of worse prognosis. Multiple genetic surveys have been performed in PTCs, however, we are not aware of any such studies in aneuploid PTCs. In order to contribute to a better comprehension of the genetic basis of this neoplasm's more aggressive behaviour in 17 aneuploid PTCs we performed a comparative genomic hybridization (CGH) analysis, studied the *BRAF* and *RAS* mutational status, searched for *RET/PTC1* and *RET/PTC3* rearrangements and determined their expression profile. Array results were validated by TaqMan and immunohistochemistry. CGH revealed multiple non-random chromosomal abnormalities. $BRAF^{V600E}$ and *RAS* mutations were found in 41.2% and 33% of the carcinomas respectively. None of the studied cases presented *RET/PTC1* or *RET/PTC3* rearrangement. When comparing array data with the chromosomal, mutational and clinical data we found that: a) loss of control of cellular transcription was of major relevance in this group of neoplasms, *HMGA2* being one of the most overexpressed genes; b) gene expression correlated with the mutational status of PTCs, as in $BRAF^{+}$ cases *cMET* and *FN1* were concomitantly overexpressed; and c) death from disease and distant metastasis was associated to the overexpression of *DDR2* and to the down-regulation of genes involved in immune, inflammatory response, signal transduction and cell adhesion processes. In conclusion we have identified in aneuploid PTCs a group of significantly altered molecules that may represent preferential targets for the development of new more efficient therapies in this type of cancer.

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Introduction

Papillary thyroid carcinomas (PTC) are the most common malignant thyroid tumours. DNA measurements by flow cytometry in PTC revealed that only a small percentage of these neoplasms (7%) were aneuploid, i.e., with a DNA index (DI) >1 (1). It was demonstrated by Sturgis *et al* (2) that aneuploidy in PTC was significantly associated with death from disease ($p<0.027$) and it has been evidenced that aneuploid nuclear DNA content was a marker of worse prognosis and should be included in the existing AMES (Age of patient, presence of distant Metastasis, Extent and Size of tumour) risk group classification (3).

In accordance with the most accepted model of thyroid tumourigenesis, papillary cancer is thought to derive through the accumulation of specific genetic alterations that cause the malignant transformation of normal follicular cells (4).

Multiple chromosomal studies have been performed in papillary carcinomas. Conventional cytogenetic analysis revealed that the most common structural chromosomal abnormalities were rearrangements with breakpoints at l0q11.2, followed by abnormalities with breakpoints at 1p32-36, 1q22, 1lp11-13, 3p25-26 and 7q32-36 (5). Comparative genomic hybridization (CGH) analysis showed that 104 of the 255 (40.8%) analysed papillary carcinomas had chromosomal imbalances. The most common gains involved regions 17q (16.3%), 1q (15.6%), 9q (12.5%), 4q and 13q (9.6%); common chromosomal losses were found in 22q (22.1%), 9q (12.5%), 13q (10.7%) and 17p (9.6%) (6-11).

Molecular studies on the genetic alterations associated to PTC pointed out that mutations in *RET, RAS* and *BRAF* genes were early, mutually exclusive, initiating events important to thyroid follicular cell transformation into papillary cancer (12). $BRAF^{V600E}$ mutations were demonstrated to be the most common of them (13). *RET* structural alterations were reported in 0-80% of the PTCs according to different studies, the most frequent being *RET/PTC1* and *RET/PTC3* rearrangements (14). RAS point mutations were described as rare in PTC but frequent and associated to aneuploidy in other follicular thyroid carcinomas (15).

Genomic analysis by expression array technologies has been used in PTC by several groups and the de-regulation of multiple growth factors and receptors, signal transduction

Table I. Clinical and histological data of the 17 analysed cases.

Case	Sex/age	Surgery date	Histological diagnosis	TNM	Disease course
1	F/88	1991	PTC, common pattern	T4N1Mx	Dead
2	F/63	1993	PTC, common pattern with PDA	T4N0M1	Persistent D.
3	F/71	1995	PTC common pattern with PDA	T4N0M0	Dead
4	F/77	1997	PTC, follicular variant	T4N1M1	Dead
5	M/64	1997	PTC, common pattern	T1bN1bM0	Remission
6	M/70	1998	PTC, common pattern	T4N1bM1	Dead
7	F/40	1998	PTC, capsulate follicular variant	T2N0M0	Remission
8	F/28	1999	PTC, common pattern	T2N0M0	Remission
9	M/75	1994	PTC, common pattern	T4N1bM1	Dead
10	F/46	2000	PTC, common pattern with PDA	T2N0M0	Persistent D.
11	F/20	2000	PTC, capsulate follicular variant	T3N0M0	Remission
12	M/56	2000	Poorly differentiated PTC	T3N0M1	Dead
13	M/59	2001	PTC, follicular variant	T3bN0M0	Persistent D.
14	F/21	2001	PTC, common pattern	T2N1M0	Remission
15	F/46	2001	PTC, common pattern	T3bN0M0	Remission
16	F/71	2001	PTC, common pattern	T1bN1M0	Remission
17	M/73	2001	PTC, tall cell variant with PDA	T4bN1M1	Dead

PTC, papillary thyroid carcinoma; PDA, poorly differentiated areas; Persistent D., persistent disease.

proteins, cell cycle regulators, tumor suppressor genes and cellular adhesion and extracellular molecules were reported to be involved in determining PTC's morphological and biological characteristics (reviewed in ref. 16).

Although PTC has been the object of several genetic studies, we are not aware of any chromosomal or expression array analysis specifically addressing the aneuploid fraction of PTC. As such, and in order to determine their genetic features and try to assess the causes of their more aggressive behaviour we performed a CGH analysis in a group of 17 aneuploid PTCs, determined the incidence *BRAF, RAS* and *RET* alterations and evaluated the expression profile of 13 of these tumours.

Materials and methods

Tumour specimens. Between 1991 and 2001, 255 tissue samples from patients with thyroid papillary carcinoma were collected for genetic studies at the Portuguese Cancer Institute in Lisbon. Of these, 17 (6.7%) had an aneuploid DNA index. It was in this fraction of tumours that we performed CGH, *BRAF, RET, RAS* and oligo-array analysis.

Histologically, all neoplasms were classified according to the most recent World Health Organization criteria for endocrine tumours (14). A summary of the clinical and histological data of this series of aneuploid PTC is depicted in Table I.

Flow cytometry analysis. DNA flow cytometric analysis was performed on fresh tissue samples using the method of Deitch *et al* (17). The ploidy status was expressed by the DNA index (DI). All tumours had a DI>1.0, as such they were defined as aneuploid (18).

CGH analysis. CGH was performed on stored liquid nitrogen fragments, after determining that each fragment comprised more than 75% of tumour cells and according to the previously described protocol (19).

Detection of BRAF, RAS and RET alterations. Evaluation of *BRAF* mutational status was performed in all aneuploid PTCs. For *BRAF* exon 15 amplification, two previously described primers were used (12). Samples presenting a restriction pattern compatible with the presence of a $BRAF^{V600E}$ mutation were further analysed by sequencing. Sequencing was performed with the ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). Data was analysed using the ABI PRISM 310 genetic analyser.

We used RT-PCR followed by cDNA sequencing to analyse *N-RAS, H-RAS*, and *K-RAS* mutations in 12 of the 17 PTC samples (Table II). Primers were designated in order to amplify PCR segments containing the mutational hot-spots of RAS genes (Table III). After purification PCR products were subjected to automatic sequencing and data analyses using the technology previously described for BRAF mutational detection.

Rearrangements of *RET/PTC1* and *RET/PTC3* were evaluated by RT-PCR, in 13 of the 17 PTCs (Table II) using the conditions and primers described elsewhere (20).

Oligonucleotide microarrays. Human thyroid total RNA from Clontech® (consisting in a pool of thyroid RNA obtained from 65 individuals which died from sudden death) was used as a normal baseline reference for the microarray experiments. Total RNA from the tumours depicted above, was extracted

Flow cytometry, *RET/PTC1* and *RET/PTC3* rearrangement, RAS, *BRAF* mutation and immunohistochemistry results aneuploid PTC.

Case	DI	*RET/PTC1*	*RET/PTC3*	RAS	*BRAF μ*	Immunohistochemistry *cMET*
1	1.72	NP	NP	NP	-	NP
2	1.97	NP	NP	NP	+	2
3	1.50	-	-	-	+	0
4	2.09	-	-	*H-RAS* Codon 61 (CAG-AAG)	-	0
5	1.97	-	-	-	+	2
6	1.92	NP	NP	-	+	2
7	1.20	-	-	-	-	0/1 (heterogeneous)
8	1.23	-	-	NP	+	4
9	1.44	-	-	-	+	2
10	1.96	-	-	*K-RAS* Codon 13 (GGC-CGC)	-	0/1 (heterogeneous)
11	1.69	-	-	-	-	0/1 (heterogeneous)
12	1.19	-	-	*N-RAS* Codon 61 (CAA-CGA)	-	NP
13	1.94	-	-	*N-RAS* Codon 61(CAA-CGA)	-	NP
14	1.09	-	-	-	+	3
15	1.15	-	-	-	-	0
16	1.42	NP	NP	NP	-	NP
17	1.79	-	-	-	-	0/1 (heterogeneous)

DI, DNA index; *BRAF μ*: *BRAF* mutation; +, mutated, -, not mutated; NP, not performed; 0, no staining; 1, weak staining; 2, moderate staining; 3, moderate/intense staining; 4, intense staining.

Table III. RT-PCR oligonucleotide primer sequences chosen to analyse *N-RAS, H-RAS* and *K-RAS* mutations.

Primer	Exon	Sequence (5'-3')
N-RAS F (forward)	2	TGCTGGTGTGAAATGACTGA
N-RAS R (reverse)	3	TCGCCTGTCCTCATGTATTG
H-RAS F (forward)	1	CGGAATATAAGCTGGTGGT
H-RAS R (reverse)	2	ATGGCAAACACACACAGGA
K-RAS F (forward)	2	GCCTGCTGAAAATGACTGAA
K-RAS R (reverse)	3	AAAGAAAGCCCTCCCCAGT

using the RNeasy mini kit (Qiagen, GmbH, Hilden, Germany) according to the manufacturer. Integrity of the total RNA was assessed in the Bioanalyser Agilent 2100 (Agilent technologies, Mill Road, PA, USA) and the concentration was determined using Nanodrop ND-1000 (Peqlab Biotechnologie, GmbH, Germany). RNA for oligo-array assays was only available from 13 of the 17 carcinomas (cases #3, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14, 15, and 17). For hybridization, the commercial GeneChip® Human Genome U133 Plus 2.0 Array from Affymetrix® (Santa Clara, CA, USA) was used. The samples were processed according to the experimental procedures specified by Affymetrix®. The obtained microarrays were scanned in GeneChip® Scanner 3000 from Affymetrix (Santa Clara, CA, USA), controlled by a workstation with the GeneChip operating software (GCOS) version 1.1.

Statistical analysis of oligo-array results. The software chosen for the analysis was the DNA-Chip analyser (dChip)® (2000-2004 Wong Lab, Harvard School of Public Health and Dana-Farber Cancer Institute, USA). In the first step of the statistical analysis the arrays were normalized with the invariant set normalization method, so that all non-biological variables were reduced, followed by a Model-based expression analysis, using the model PM-only. The genes were filtered, so that those absent in all the samples were eliminated from the analysis. We considered genes to be differentially expressed between samples, when the lower bound of fold change (LBFC) was at least 2, with a confidence of 90%. The obtained data was subject to hierarchical clustering and principal components analysis (PCA). The genes were also classified according to 'gene ontology' using the gene classifying tool of

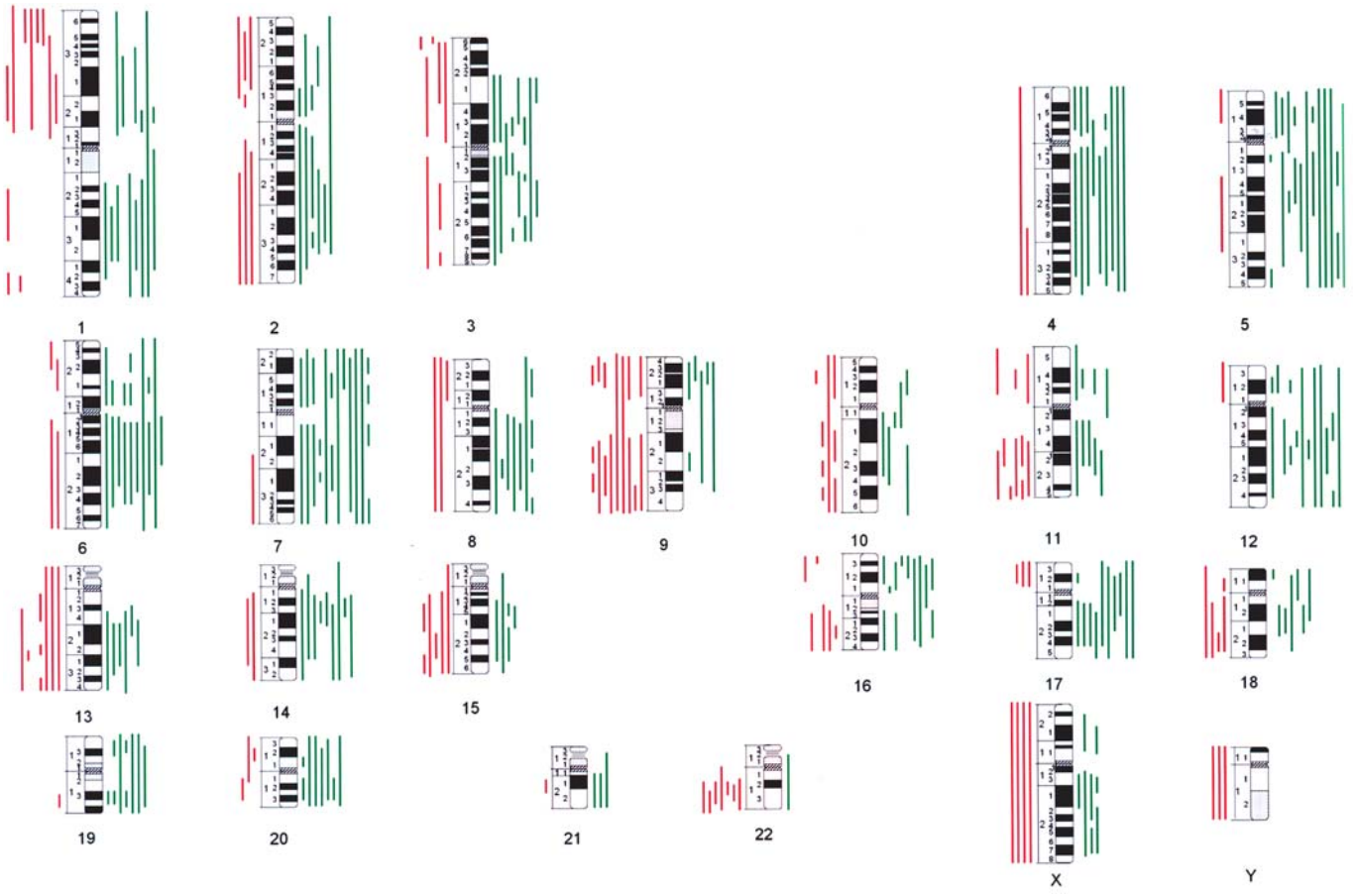

Figure 1. (A) Summary of the DNA copy number changes detected by CGH. Each bar on the left side of the chromosome ideograms (red) represents a loss in one tumour and each bar on the right side (green) represents a gain in one tumour.

D-Chip. We regarded only the p significant genes ($p<0.001$) for this classification.

Quantitative real-time RT-PCR. To validate the array results, quantitative real-time RT-PCR was performed on 4 genes: fibronectin 1 (*FN1*), met proto-oncogene (hepatocyte growth factor receptor) (*cMET*), chemokine (C-X-C motif) ligand 14 (*CXCL14*), and discoidin domain receptor 2 (*DDR2*) using an ABI PRISM 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA). Sequence specific primers and probes were selected from the Assay-on-Demand products (Applied Biosystems). As endogenous control we selected the housekeeping gene *eukaryotic 18s rRNA* (Applied Biosystems). The conditions of the TaqMan® PCR were as follows: 95˚C for 10 min, followed by 45-50 cycles of 95˚C for 15 sec and 60˚C for 1 min. The relative expression of each sample was calculated with respect to a standard calibration curve that represents a serial dilution of cDNA positive for the expression of the gene in analysis. Each sample was analysed three times and each PCR experiment included at least one non-template control well.

Immunohistochemistry. We performed immunohistochemistry assays on paraffin-embedded tissues for *cMET* proto-oncogene in 13 of the 17 PTCs (Table II). Sections with 2-3 μm thickness were cut from formalin-fixed paraffin-embedded thyroid tissue blocks and stained with hematoxylin and eosin to confirm the histological diagnosis. To enhance antigen exposure, specimens were heated using a microwave oven in a 600 ml sodium citrate buffer (0.01 M, pH 6.0), Slides were incubated overnight at 4˚C with a rabbit polyclonal antibody directed against *cMET* (Zymed, San Francisco, CA). An anti-rabbit Envision polymer (Dako, Glostrup) was applied for 30 min followed by a chromogenic substrate (3,3'-diaminobenzidine, Dako) for 10 min. Sections were counterstained with Mayer hematoxylin for 5 min. Negative controls were carried out by omitting the primary antibody and a papillary carcinoma known to express *cMET* was used as positive control. Staining intensity was evaluated semi-quantitatively by an expert thyroid pathologist. The intensity of *cMET* staining was graded on a scale from 0 to 4: 0, absent; 1, weak; 2, moderate; 3, moderate/ intense; and 4, intense. In some cases, there were areas with different staining intensities. These cases were classified as heterogeneous.

Results

Flow cytometry analysis. The DNA indexes (DI) for the 17 cases are depicted in Table II. The assessed DI of the tumours varied between 1.09 and 2.09, as such all tumours were considered to be aneuploid.

CGH analysis. CGH results are summarized in Fig. 1. CGH imbalances were observed in all 17 PTCs. The most frequent DNA gains involved the 5q region in 70.6% of the cases,

by gains of 7p, 7q and 12q in 64.7%, at 5p, 6q, and .8% and at 4q, 14q and 16p in 52.9% of the tumours. None of the PTC presented high level amplifications. Chromosomal losses were also frequently observed. By order of frequency, losses were at 9q in 52.9% of the cases, at 1p in 47%, at 9p and 22q in 41.2% and at 11q and 13q in 35.3% of the neoplasms.

Detection of BRAF, RAS and RET alterations. $BRAF^{V600E}$ mutation was observed in 7 of the 17 (41.2%) aneuploid PTCs (Table II).

RAS mutations were detected in only 4 of the 12 (33%) PTC samples (Table II). Two of four mutations were in codon 61 of *N-RAS*, one in codon 61 of *H-RAS*, and one in codon 13 of *K-RAS*. Mutations found in *N-RAS* were transitions AT→GC at the second nucleotide of CAA codon resulting in a *Gln→Arg* substitution, the mutation found in *H-RAS* was a transversion CG→AT at the first nucleotide of CAG codon (*Gln→Lys* substitution) and the mutation found in *K-RAS* was a transversion GC→CG at the first nucleotide of GGC codon (*Gly→Arg* substitution).

None of the 13 analysed cases presented *RET/PTC1* or *RET/PTC3* rearrangements (Table II).

Oligonucleotide microarrays. Since papillary cancer is thought to arise *de novo* through the malignant transformation of normal follicular cells (4) assessment of the expression profile of this aneuploid group of PTCs was performed, by comparing the array data from the 13 selected tumours, with the expression array data obtained for normal thyroid (NTHY). We found significant differences ($p<0.0001$) in the expression of 4674 probe sets. The 10 most overexpressed and underexpressed genes identified when comparing tumours versus NTHY, are shown in Fig. 2A. Ontologically the differentially expressed genes were grouped in 2 main sets: regulation of transcription DNA-dependent (59%), and extracellular matrix (10%) (data not shown).

In this group of aneuploid PTCs *BRAF* and *RAS* mutations were detected in 7 and 4 cases respectively. By hierarchical clustering we found that carcinomas with and without mutations had distinct genomic profiles (Fig. 3A and B). PCA analysis of PTC log transformed data for all probe sets of PTCs with reference to their gene mutational status (Fig. 3C) revealed a clear segregation between $BRAF^+$ (n=5) and RAS^+ (n=4) cases, demonstrating a relationship between gene expression and the carcinomas mutational status. Corroborating previous data (21) we verified that in $BRAF^+$ neoplasms signal transduction occurred preferentially through MAPK pathway. In fact only in this set of PTCs *MET* and *FN1* were overexpressed. The LBFC for *cMET* was 2.82 and for *FN1* it was 2.93.

In order to assess the genetic basis of a more aggressive behaviour in aneuploid PTC we compared the array data of patients who had distant metastasis at diagnosis (Ml) and were known to carry a worse prognosis versus those who did not (MO), and of patients who died from the disease versus those in remission.

Accordingly, when comparing M1 (n=4) versus M0 (n=9) cases, we found 227 differentially expressed probe sets between the two groups. Almost all probe sets (219) were down-regulated in the M1 carcinomas. Hierarchical clustering of M1 vs. M0 tumour samples allowed a clear segregation between the two groups (Fig. 2B). There were only 4 genes more expressed in M1 cases. These genes were discoidin domain receptor family, member 2 (*DDR2*); NADH dehydrogenase (ubiquinone) 1, subcomplex unknown, 2, 14.5 kDa (*NDUFC2*); the hypothetical protein BC000993; and the GNAS complex locus. *DDR2* was the gene presenting the highest LBFC (LBFC=8.6). The three most underexpressed genes in M1 carcinomas were immunoglobulin genes (Fig. 2B). By gene ontology analysis (data not shown) it was also observed that most of the down-regulated genes in M1 PTCs were immune and inflammatory response genes (34%) followed by a cluster of genes identified as 'integral to plasma membrane' (23%) that included molecules involved in signal transduction and adhesion processes. One of these genes was claudin 16 (Fig. 2B).

When we compared the array data from the patients that had died from the disease (n=4) (we excluded patient 3 from this analysis, as she died from complications at surgery) with data from patients in remission (n=6) we found 154 differentially expressed probe sets, most of which were (137) down-regulated in the group of patients that died. Fig. 2C is a dendrogram of the hierarchical clustering between death vs. remission cases. A clear segregation between the two groups was observed and we found that 4 immunoglobulin genes were among the 10 most underexpressed genes in the set of fatal PTC cases (Fig. 2C). Ontologically (data not shown) we verified that in this group of patients the differentially expressed genes were grouped in 4 main sets: immune and inflammatory response (35%) and proteins known to be involved in signal transduction and cell adhesion (23%). *DDR2* was one of the most overexpressed genes in this group of patients (Fig. 2C).

Quantitative real-time RT-PCR. The results of the arrays were validated by quantitative real-time RT-PCR, using 4 genes relevant in distinguishing groups of tumours: i) *cMET* and *FN1* which were overexpressed only in $BRAF^+$ cases and which are known effectors of the MAPK signalling pathway; ii) *CXCL14* whose loss of function was proposed to contribute to immunological escape in several tumour types (22) and which was underexpressed in cases presenting distant metastasis; and iii) *DDR2* which was one of the few overexpressed in cases presenting distant metastasis, in the group of patients that died from disease and was also overrepresented when all carcinomas were compared with normal thyroid.

For all these genes the levels of expression observed by quantitative RT-PCR correlated with the data obtained by microarray analysis (Fig. 4A-F).

Immunohistochemistry. Immunohistochemistry analysis of the *cMET* protein expression in 13 aneuploid papillary cases revealed that there was a statistically significant association between *BRAF* mutation and increase of *cMET* expression ($p=0.0047$) at the protein level. Six out of 7 cases presenting *BRAF* mutation showed a moderate, moderate/intense or intense staining for *cMET*. The 6 cases without *BRAF* mutation had a negative staining or were heterogeneous, showing regions without and others with a weak staining for *cMET* (Table II, Fig. 4G-I).

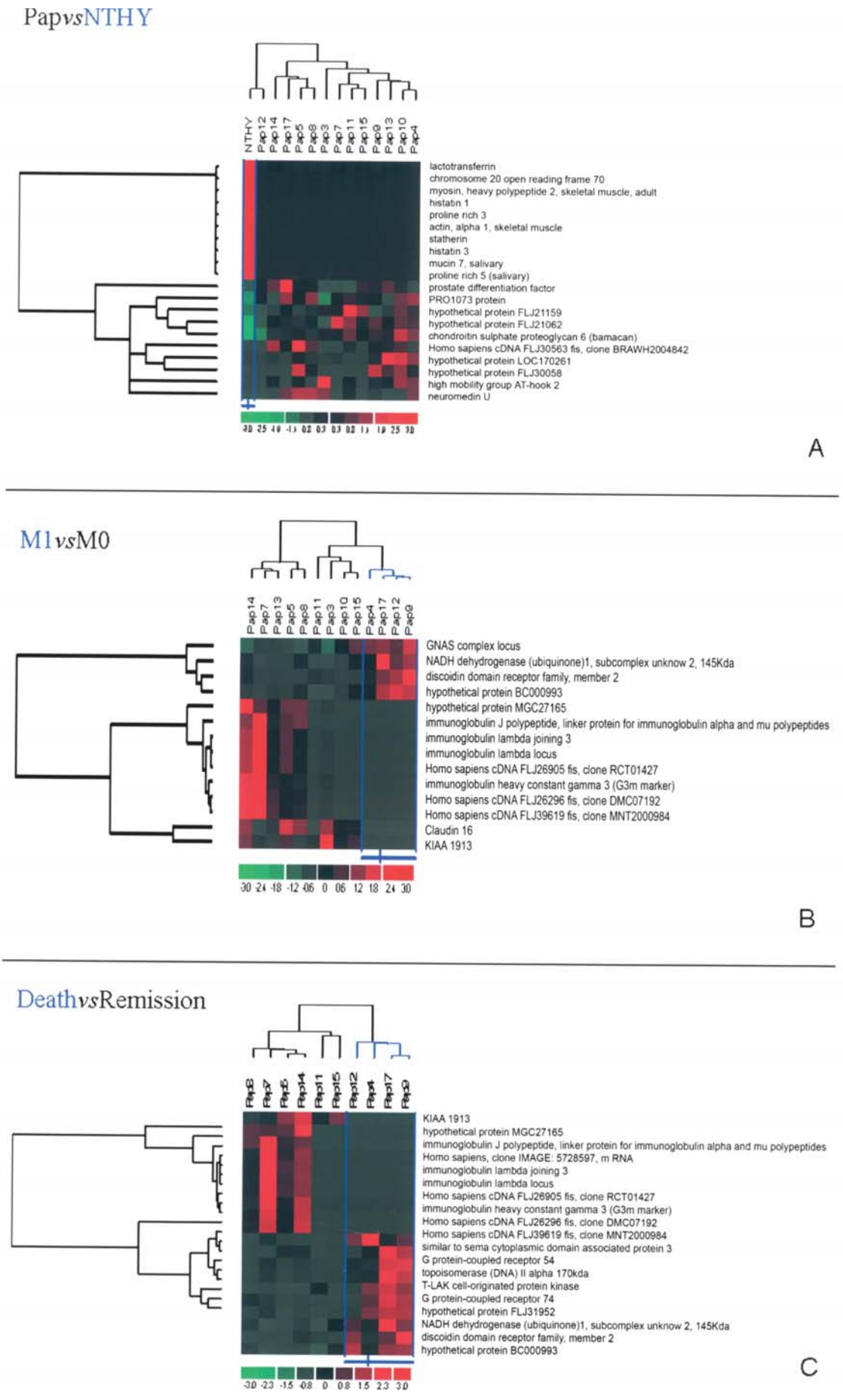

Figure 2. Dendrogram of the hierarchical clustering using the 10 most overexpressed and underexpressed genes when comparing (A) the 13 aneuploid PTC vs. normal thyroid (NTHY), (B) M1 vs. M0 cases (only 4 overexpressed genes were found in M1 cases and used for clustering), and (C) patients who died from disease vs. those in remission.

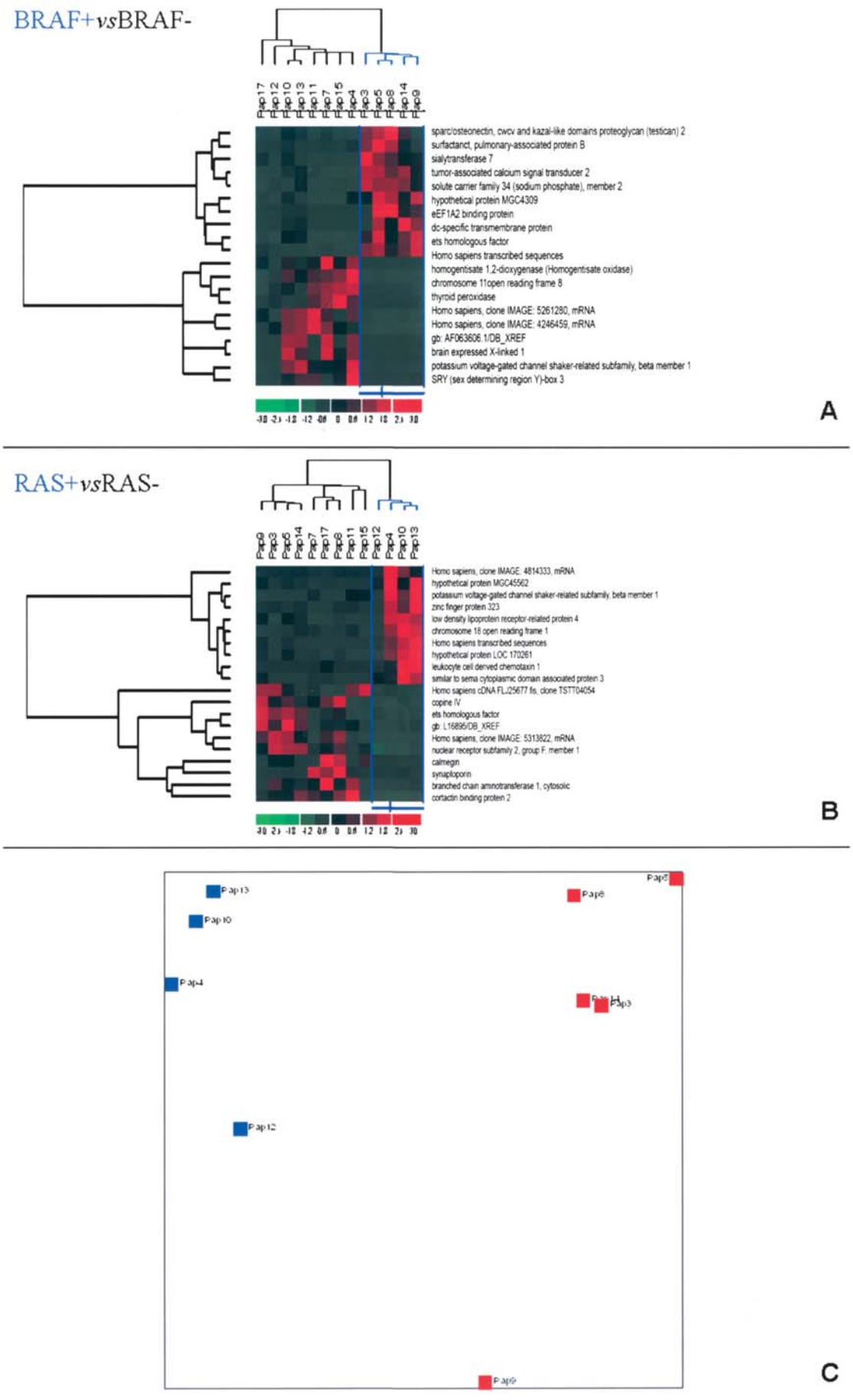

Figure 3. Dendrogram of the hierarchical clustering using the 10 most overexpressed and underexpressed genes when comparing (A) *BRAF*$^{+}$ vs. *BRAF*$^{-}$ cases, (B) *RAS*$^{+}$ vs. *RAS*$^{-}$ carcinomas, and (C) PCA analysis of PTC log transformed data of all probe sets of PTCs with reference to their BRAF and RAS mutated status. In red are represented the *BRAF*$^{+}$ and in blue the *RAS*$^{+}$ carcinomas.

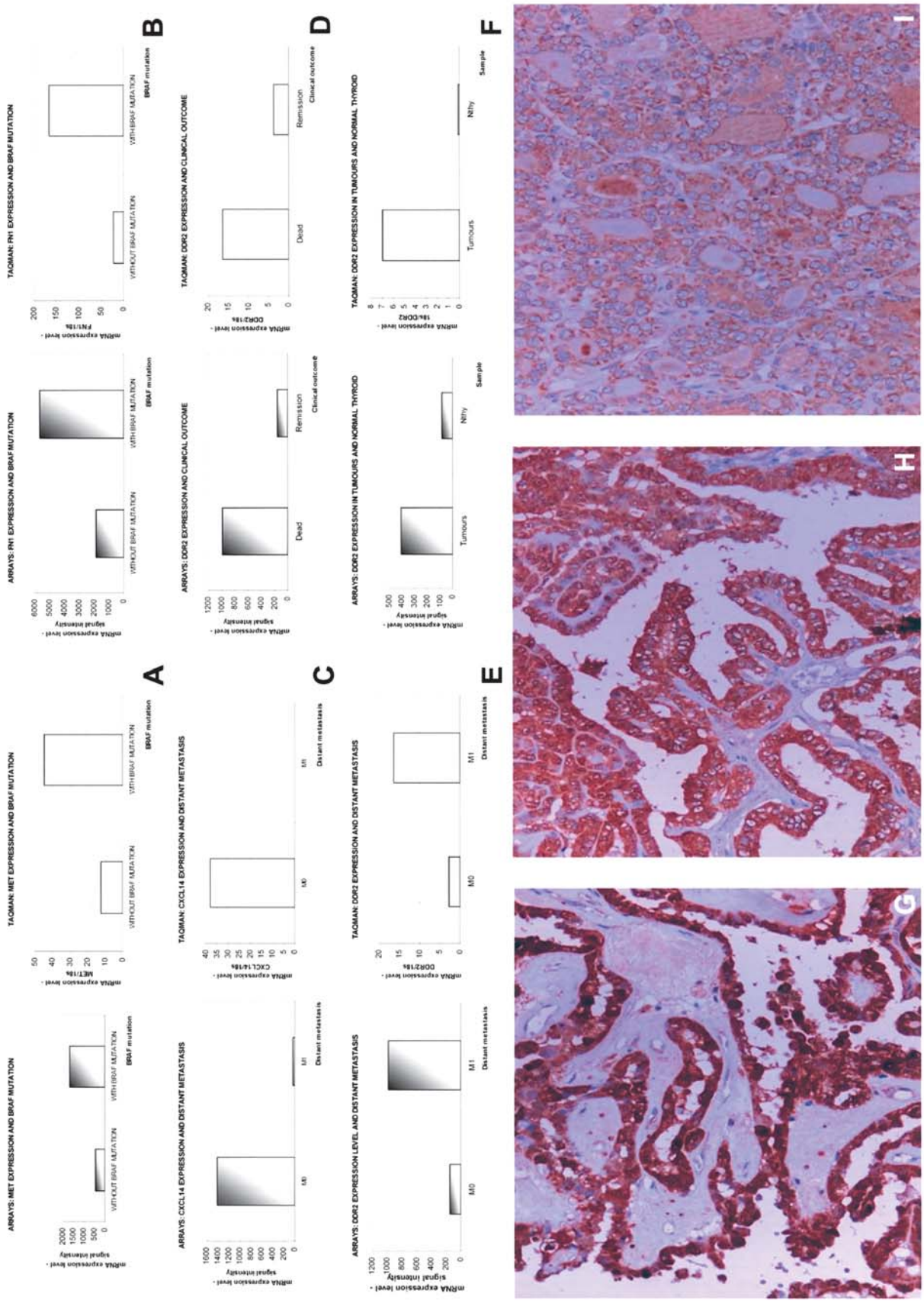

Figure 4. Comparison of RNA assays by microarray hybridization (measured by signal intensity), graphs on the left side; and by real-time quantitative RT-PCR/TaqMan, (measured by gene/18s), graphs on the right side. (A) Comparison of cMET expression between cases with and without BRAF mutation, (B) comparison of *FN1* expression between cases with and without BRAF mutation, (C) comparison of *CXCL14* expression between cases with and without distant metastasis, (D) comparison of DDR2 expression between dead and alive patients, (E) between M1 and M0 cases, (F) and between tumour samples and normal thyroid. Immunostaining for cMET in (G) case 8 ($BRAF^+$): intense staining (x200), (H) case 9 ($BRAF^+$): moderate staining (x200), (I) case 11 ($BRAF^-$): heterogenous weak/no staining (x100).

Several genetic studies have been performed in PTC, however, to our knowledge, our study represents the first that specifically addressed the aneuploid fraction of papillary tumours.

CGH analysis of the 17 PTC revealed multiple chromosomal imbalances in all cases. In this series of tumours we found that, similarly to what is observed in follicular thyroid adenomas and carcinomas (19), aneuploid PTC presented frequent gains involving 5p/5q, 7p/7q, 12q and 17q. However, at variance with follicular type neoplasms, they were characterized by a high frequency of gains at 6q and losses at 1p, 9p/9q, and 22q. Kjellman *et al* (8) reported that loss of 9q21.3-q32 and gain of 1q were exclusively seen in PTC from patients with aggressive disease, and the presence of distant metastasis was associated with gains of 1q. In our set of 17 aneuploid PTC, we did not find this association. Indeed, we found 1q gain in 8 of our cases (47%); of those, in the 7 where TNM classification was attainable, only 3 presented distant metastasis.

Analysis of the expression profiles of the 13 aneuploid PTC revealed, in keeping with the great chromosomal instability that characterized this set of tumours, an extensive alteration of the gene expression levels. When compared to NTHY we found significant differences ($p<0.0001$) in the expression of 4674 probe sets. Ontologically we identified that most of these differentially expressed probe sets (59%) were regulators of transcription DNA-dependent genes. This data suggests that loss of control of cellular transcription is of major relevance in the tumourigenesis of aneuploid PTC. Accordingly, we found that one of the 10 most overexpressed genes in this series (Fig. 3A) was *HMGA2*, a gene mapped to chromosome 12q15 and that codes for an architectural transcription factor which is an essential component of the enhancement. The involvement of *HMGA2* in thyroid tumourigenesis has been previously demonstrated. Accordingly, Berlingieri *et al* (23) reported a high expression of *HMGA2* in malignant cell transformation in rat thyroid cells, and in human thyroid cancer. Scala *et al* (24) demonstrated that gene transfer of an antisense adenoviral vector for the high mobility group of proteins induced programmed cell death in two human thyroid anaplastic cell lines: ARO and FB-1, but not in normal human thyroid cells.

The incidence of *BRAF, RET* and *RAS* alterations has never been evaluated in aneuploid PTC. *BRAFV600E* mutation was observed in 7 of the 17 cases (41.2%). The detected frequency of *BRAF* mutations is similar to that reported by others (13) and as previously observed none of our follicular variant PTCs were *BRAF*$^+$. In our series no *RET/PTC1* and *RET/PTC3* rearrangements were found. Fagin (15) advanced the existence of a relationship between the presence of RAS mutations and aneuploidy in follicular type carcinomas, and Saavedra *et al* (25) verified that the introduction of oncogenic *H-RAS* into the PCCL3 thyroid cell line induced chromosomal instability. Although, we could not study all 17 carcinomas due to sample insufficiency the observation that only 4 of the 12 PTCs had RAS activating mutations indicates that in this fraction of papillary carcinomas oncogenic RAS transformation is not a determinant modification for aneuploidy to occur. However, two of the RAS$^+$ cases were follicular variant PTCs (Table II).

In a previous microarray analysis of a pool of 40 PTCs Giordano *et al* (21) demonstrated the existence of a mutational specific expression signature associated to *RET/PTC, RAS* and *BRAF* mutation groups. No reference was made to the ploidy status of the tumours in their report, but it is noteworthy that also in our aneuploid PTC we verified that the *BRAF*$^+$ and *RAS*$^+$ carcinomas had different and specific expression profiles (Fig. 3A-C). Our findings evidence that although in a complex genetic background the occurrence of dominant activating mutations in *BRAF* and *RAS* has a fundamental role in the set up of gene expression alterations.

Giordano *et al* (21) have also remarked that *BRAF*$^+$ mutants signalled preferentially through MAPK pathway, *MET* and *FN1* being included in the group of the 10 most overexpressed genes in their study. In our set of BRAF$^+$ carcinomas these two genes were not in the list of the 10 genes with the highest LBFC. However, we noticed their up-regulation only in this group of carcinomas. Quantitative real-time RT-PCR data and immunohistochemistry experiments confirmed our array results (Fig. 4A, B, and G-I) with a statistically significant association between *BRAF* mutation and increase of *cMET* expression ($p=0.0047$) at the protein level. Our data allows us therefore to conclude that also in aneuploid PTCs BRAF$^+$ tumours signal through the MAPK pathway. Considering that in a near future molecularly driven therapies will preferentially be applied in the treatment of cancer our findings are highly suggestive that cMET may be one of the target genes to consider in all types of BRAF$^+$ carcinomas independently of their ploidy status.

When we evaluated for the expression profile alterations according to clinical data, we were able to verify that 98% of the differentially expressed genes between M1 and M0 PTCs were down-regulated in the M1 group and that most of the underexpressed genes were involved in immune and inflammatory response, signal transduction and cell adhesion processes. Remarkably, when we compared the expression profiles of patients who died vs. those in remission we also observed that 89% of the differentially expressed genes were down-regulated in patients who died from the disease and that they were clustered through the same main ontological molecular pathways as in the M1 patients. These findings strongly suggest that in this aneuploid group of carcinomas down-regulation of genes involved in these four cellular mechanisms is one of the genetic causes of a more aggressive tumour behaviour.

Among the very few overexpressed genes in the M1 and the death groups of patients we verified that *DDR2* was up-regulated in both. *DDR2* is a tyrosine kinase receptor whose activating ligands are collagens. DDR proteins are involved in the proliferation of several cell types, and their induction is implicated in breast and ovarian cancer, correlating to metastasis (26,27). The role of *DDR2* was never investigated in thyroid neoplasias, however, our data points out that this molecule should be further evaluated in order to test its putative role in aneuploid PTC cancer mechanisms, namely those conducing to a higher aggressive behaviour.

In summary, we were able to identify in a group of PTCs with a more aggressive tumour behaviour a number of significantly altered molecules that are involved in processes such as regulation of cell transcription, cell adhesion, signal

transduction, and immune and inflammatory responses and that represent preferential targets for the development of more efficient therapies.

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