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PAPILLARY THYROID MICROCARCINOMA: ASSOCIATION BETWEEN AGGRESSIVENESS INDICATORS AND PROGNOSTIC FACTORS IN A HIGH PREVALENCE OF GOITER AREA

Tesi di

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Introduction

Thyroid carcinomas represent about 90% of endocrine malignancies (1); their are divided into papillary carcinoma (PTC), follicular carcinoma (FC), medullary thyroid carcinoma (MTC), anaplastic thyroid carcinoma (ATC) (2).

The papillary thyroid carcinoma (PTC) is the most common malignant thyroid tumor, representing 80-90% of thyroid malignancies (1, 2); it is the more frequent in childhood and < 50 years (2).

The data collected at the Surveillance Epidemiology and End-Results Cancer Registries program (SEER) indicate an annual average prevalence of thyroid carcinoma of 6.6/100.000 with an annual increase > 5% in the period 1975-2002. This increase is mainly due to an improved diagnostic histopathology with a more frequent use the cytological diagnosis on fine needle byopsy (2). Furthermore, between 1988 and 2002, a 49% increase in tumors equal to or less than 1 cm was noted; it has been postulated that at least part of this rise of papillary thyroid microcarcinoma (PTMC) is related to increased detection

primarily due to the wide use of thyroid ultrasound, which allows the detection of small nonpalpable nodules (3).

The papillary thyroid microcarcinoma (PTMC), according to the World Health Organization (WHO), is defined a tumor mesuring 1 cm or less in diameter (1, 3, 4, 5, 6), it may be solitary, or multiple microfoci may be present (3).

In 1997, Moosa and Mazzaferri defined "occult thyroid carcinoma "as an "unpalpable thyroid carcinoma that is generally smaller than 1 cm" (7). A more precise definition of size is used by Stedman's Medical Dictionary (2006), where occult papillary carcinoma of the thyroid is described as microcarcinoma of the thyroid or microscopic papillary carcinoma of the thyroid, usually well encapsulated and measuring less than 5 mm in diameter (7,8).

A combination is used in the WHO (World Health Organization) classification system, where papillary thyroid microcarcinoma (PTMC), as mentioned, is defined as "papillary carcinoma measuring 1cm or less in maximal diameter while other clinico-pathological features, such as metastasis to regional lymph nodes and/or distant organs as well as extrathyroid extension,

are not considered" (9).

The reported prevalence of multifocality appears to be present in 30-40% of cases, whereas bilateral disease is found in approximately 20% of involved glands. Cervical lymph node adenopathy is discovered in 25-43% of patients with PTMC. Extrathyroidal extension is noted in about 15-21%, whereas vascular invasion may be present in approximately 3.5%. Distant metastases occur unfrequently at a rate of 1-2,8% (3).

It could be defined as a thyroid incidentaloma, in fact it is occasionally identified at US of the neck performed for other reasons but its diagnosis is often incidental (2,3).

Autopsy studies have revealed that the occurrence of microcarcinoma, primarily foci of PTC, ranges between 6 and 36% according some authors (3) and 0.01% in USA and 35.6% in Finland (the highest value reported in the literature) according to other authors (10).

In Western countries and in Japan the percentage of papillary thyroid carcinoma is increasing, from 78.4% thirty years ago to 93%, registered by the Japanese Society of Thyroid Surgeons

(JSTS), in 2004. A similar situation has been reported in Western countries, where, in 2002, papillary carcinoma comprised 85.3%. The prevalence of incidentally detected PTMC found at autopsy and from clinical studies was 100-1000-fold higher than the incidence of clinical cancer (11,12). This finding strongly suggests that most papillary microcarcinomas remain latent and do not become clinically apparent. The determination of clinical and especially molecular parameters which would define a small group of PTMC with an aggressive biological behaviour is foundamental (7).

The annual rate mortality of PTMC is between 0.4-2.8 and 0.2-12/100.000, respectively, for women and men (2).

The clinical behavior of PTMC is usually indolent as demonstrated by recent literature; in fact in a recent work, Hoon Y.K et al., emphasizes that one series of 93% of patients were free of disease during a follow-up of 3-23 years, with a mean of 6.3 years, and there were no distant metasteses.

However, rarely PTMC may have aggressive behavior with locoreginal recurrence and cervical lymph node metastases. The

lack of long-term prospective randomized studies makes it very difficult to establish which therapeuthic approach is better and explains the present uncertainty and controversies for the management of PTMC (1).

Its etiology is the result of complex genetic and environmental factors interaction in individuals at risk (2).

Probably the most important risk factor is genetic predisposition, which is supported by the fact that the prevalence in the Japanese population exposed to the radiation during the bomb attack on Hiroshima and Nagasaki (11.3-28.4%) (13,14,15) and in the Japanese population staying in Hawaii, without the radiation exposure (24%) (15,16), is similar.

In the literature, several critical genetic alterations, associated with development of specific thyroid tumour types, have been described. The three types of genetic alteration in the Mitogen-activated Protein Kinase (MAPK) pathway, including RET rearrangement and Ras and BRAF mutation, are present in approximately 70% of PTCs (17).

In a recent study the authors (18) showed that the PTMC had clinical and pathological factors related to prognosis, evaluated

with multivariate analysis (18).

The aggressive behavior of PTMC may be related to the molecular alterations.

Starting with an analysis of the literature and of our experience we want analyze the association betwen aggressiveness indicators (extracapsular spread, metastatic lymph nodes) and prognostic factors (age, tumor size, uni/multifocal disease,) in a high prevalence of goiter area.

Risk factors for thyroid cancer

Iodine deficiency

Iodine intake is mentioned as an important environmental factor. A wide range of variants has been found in different parts of the world. In areas with sufficient iodine intake, the incidence of thyroid cancer is higher and a predominance of papillary carcinoma has been mentioned. In 2005, Kovacs et al. (11) published results of a study, where consecutive series of autopsies were performed in Hungary in two areas with different iodine uptake (19). They concluded, that the prevalence of microcarcinomas was not related to iodine intake, because from

222 thyroids examined in an iodine deficient group and from 221 thyroids in an iodine sufficient group, the prevalence was only 4.74%, with respect to 4.52% (11).

Exposure to radiation

The thyroid gland is highly sensitive to radiation inducing oncogenesis and both types of radiation, external or internal (delivered from radioiodine), are the most prominent factors in the development of thyroid cancer. The most vulnerable is the thyroid gland in children, which was clearly documented in Belarus et in Ukraine, in connection with the Chernobyl accident (7).

The incidence of thyroid cancer, in children, was less than 1 per million, per year, before the accident, but after the accident increased, in certain areas, to 100 cases per million per year. Surprisingly, in the Chernobyl region, a big difference was found in children, exposed to radiation in the age group younger than 3 years and those exposed before birth (in utero), born after the accident. Fifteen years after the accident, in the first group, 33 cases of thyroid cancer were found (out of 9472 children),

compared to no cases in the group exposed in utero (out of 12129 children) (7).

Environmental factors in Sicily

In a recent study Pellegriti et al. have reported a substantially higher incidence rate of thyroid cancer in the Catania province of Sicily than in other Sicilian provinces and in other parts of Italy. They also explored associations of a range of possible risk factors for thyroid cancer with the presence of the volcano Mount Etna (which is in close proximity to the town of Catania) (20).The incidence rates of thyroid cancer is increasing more rapidly than that of other cancers in the United States and its incidence is increasing in many other countries, including France and Italy. The average incidence of thyroid cancer among women and men is 14.1 and 4.9 diagnoses per 100.000 residents per year, respectively, in the white population of the United States and 12.9 and 5.0 diagnoses per 100.000 residents per year, respectively, in Europe. In Italy, the average incidence among women and men is 15.5 and 5.2 diagnoses per 100.000

residents per year, respectively. Many experts, as mentioned, attribute this increasing incidence to more intensive and sensitive diagnostic procedures to detect thyroid nodules and to diagnose microcarcinomas. Environmental carcinogens associated with an industrialized lifestyle might contribute to the increasing thyroid cancer incidence. Until now, however, the only environmental factors that have been strongly associated with thyroid cancer are exposure to radiation and iodine deficiency . An increased incidence of thyroid cancer has also been reported in volcanic areas with basaltic characteristics, such as the volcanic regions of Iceland and Hawaii . The Mt Etna volcano in Sicily also has basaltic characteristics and hosts a major aquifer that provides drinking water to more than 750.000 inhabitants and irrigation to large agricultural areas nearby. Water from this aquifer and the volcanic soil undergo a magmatic-type interaction, in which excess CO_2 in volcanic gas leads to acidification of water and to leaching of chemicals from the basalt rock, especially on the lower south-southwestern and eastern flanks of the volcano. Various elements and chemicals (including HCO_3, SO_4, calcium, fluoride, chloride, magnesium,

boron, manganese, iron, and vanadium and their salts and also ^{222}radon) are often increased in water samples from various sources of this volcanic aquifer, as reported in studies of water samples in other volcanic areas. The Sicily Island has rural and urban areas, industrial and nonindustrial areas, areas of low and adequate iodine intake, volcanic and nonvolcanic areas, and a homogenous population; thus, Sicily is a favorable setting for the evaluation of environmental influences on thyroid cancer etiology.

This data are collected in The Sicilian Regional Registry for Thyroid Cancer; it was instituted in January 2002 (21).

BRAF

BRAF gene encodes a protein belonging to the family of serine-threonine kinases, activator of mitogen-activated protein kinase (MAPK) with a high affinity for MEK1 and MEK2, MAP kinases, leading to their phosphorylation more efficiently than other RAF isoforms.

MAPKs respond to mitogenic extracellular stimuli and regulate gene expression, mitosis, differentiation, proliferation, and cell servival/apoptosis (2).

The MAPK pathway is activated by signals from a variety of cell surface receptors and growth factors (22).

MEK and MEK2 activate the serine/threonine specific protein kinases ERK1 and ERK2. Activated ERKs are pleiotropic effectors of cell physiology and play an important role in the control of gene expression involved in the division cycle, apoptosis, cell differentiation, and cell migration.

BRAF mutation is the most common genetic alteration found in PTCs (approximately 45% of these tumors); their mutation is present in 40-70% of PTCs with higher percentage of positivity in more aggressive variants such as " tall cell " dedifferentiated forms (2).

This mutation is detected in 15.8%–52% of PTMC cases (5).

In particular, the genetic alteration (point mutation) with the higher prevalence in classical PTCs involves the nucleotide 1799 determining a valine-glutamate substitution at amino acid

residue 600 (V600E) with consequent activation of BRAF kinase that results in a contiuous phosphorylation of MEK and MAPK pathway effectors. Such a mutation is very rare in FTC (23,24, 25).

The characteristics of aggressiveness of PTCs, such as extrathyroidal extension, advanced presentation, presence of lymph node, or distant metastases have been associated in many studies with presence of BRAF mutation (26, 27).

In the studies on transgenic mice with thyroid-specific expression of BRAF V600E, they developed a PTC with invasion of blood vessels, thyroid capsule, and perithyroid skeletal muscle. These characteristics are all features of aggressiveness , demonstrating a progression to poorly differentiated thyroid carcinomas (2).

The BRAF mutation plays a fundamental role in progression, invasiveness and recurrence of PTC; it is also associated with overexpression of the Vascular Endothelial Growth Factor (VEGF) (28), which is a strong angiogenic protumour molecule that plays a critical role in human cancer progression and

invasion (29) . The explanation for this proangiogenic BRAF – VEGF association is in the unique molecular mechanism, when BRAF mutation promotes VEGF overexpression and inhibition of Tissue Inhibitor of Metalloproteinases-3 (TIMP3) (30).

TIMP3 is the tumour inhibitor, which suppresses tumour growth, angiogenesis, invasion and metastasis by preventing the interstitial matrix destruction promoted by matrix metalloproteinase 3 (MMP-3). Moreover, BRAF mutation, in the primary PTC, is associated with loss of radioiodine avidity in the recurrent tumour (2).

The BRAF mutation in studies from Korea reported an unusually high prevalence in both PTC (80-90%) and PTMC (65%) (7).

Hoon You Kim et al. analyzed the gene expression profiles of PTMC, never been reported before, and compared those with PTC. Most of the commonly up-regulated and down-regulated genes in PTMC were functionally associated cells adhesion and cells-metiated immumity. PTMC and PTC show no difference in gene expression level. For the authors, thus, the PTMC should not be considered as the simple occult indolent cancer but as the

earlier stage of diseases with evolves into PTC (1).

Was recently reported that BRAF mutations enhance the capacity of BRAF mutated cells to proliferate and transform. It has also been suggested that lymph node metastases of papillary cancer are accompanied by a new BRAF mutation, different from that observed in the matched primary thyroid cancer, confirming the progression model of cancer where metastatic foci have a new mutational event . These results suggesting that PTMC harboring an activating mutation of the gene for BRAF might have a more aggressive behavior have not been confirmed by another study in Korean patients (10).

Materials and Methods

We performed this study from the Service of Endocrine Surgery, Unit of General and Emergency Surgery, Department of General, Emergency and Transplant Surgery, University of Palermo (Italy), in which, from january 2003 and december 2010, 1211 total thyroidectomies or completion thyroidectomies were performed. Medical records were examined and 424

malignancies were found. Off all, 343 were papillary carcinomas. Among them, 141 were ≤ 1 cm. The preoperative diagnosis was done with systematic use of ultrasonography integrated with color-Doppler. Ultrasonographic criteria for suspected diagnosis of PTMC were: microcalcifications; hypoechoic pattern; increased nodular vascularity; infiltrative margins (no "halo sign"); taller than wide on transverse view. All suspected nodules were studied with a Ultrasound-Guided Fine-Needle Aspiration Biopsy (FNAB); the samples were analyzed for cytology. Cytopathological findings were classified according to Bethesda system (31) (Tab. 1).

Table 1:The Bethesda System for Reporting Thyroid Cytopathology

I.	**Nondiagnostic or Unsatisfactory** Cyst fluid only Other (obscuring blood, clotting artifact, etc)
II.	**Benign** Consistent with a benign follicular nodule (includes adenomatoid nodule, colloid nodule, etc) Consistent with lymphocytic (Hashimoto) thyroiditis in the proper clinical context Consistent with granulomatous (subacute) thyroiditis Other
III.	**Atypia of Undetermined Significance or Follicular Lesion of Undetermined Significance**
IV.	**Follicular Neoplasm or Suspicious for a Follicular Neoplasm** Specify if Hürthle cell (oncocytic) type
V.	**Suspicious for Malignancy** Suspicious for papillary carcinoma Suspicious for medullary carcinoma Suspicious for metastatic carcinoma Suspicious for lymphoma Other
VI.	**Malignant** Papillary thyroid carcinoma Poorly differentiated carcinoma Medullary thyroid carcinoma Undifferentiated (anaplastic) carcinoma Squamous cell carcinoma Carcinoma with mixed features (specify) Metastatic carcinoma

Non-Hodgkin lymphoma
Other

In the patients treated since 2006, if a fine-needle aspiration biopsy had been preoperatively performed, the B-RAF V600E were investigated in the sample. The pellet was centrifugated and the genomic DNA was drawn out using a Quiagen DNAeasy Tissue Kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). BRAFV600E mutation was detected by real-time allele-specific amplification as described elsewhere (32). As described in the literature (32) amplification was performed with a Lightcycler (Roche Diagnostics GmbH, Mannheim, Germany), and fluorescence was measured. After completion of the cycling process, samples were subjected to melting curve analysis. Positive results were further confirmed using a B-RAF Mutector Single-Well Test Kit (Trimgen, Tebu-Bio, Milan, Italy), following the manufacturer's instructions. According to original paper of Pizzolanti and coll., cases in which the genotype ratio (absorbance from sample=absorbance from wild type) was ≥2 were interpreted as mutant (33). Genomic DNA was extracted using the QIAamp Tissue Kit

(Qiagen, Hilden, Germany) with the standard protocol, and BRAF mutation detected as above described. RNA extraction from ex vivo thyroid nodules . After surgical resection, thyroid samples from nodules obtained by means of careful identification by an experienced pathologist and corresponding to the biopsied nodule were quickly stored in RNA later (Sigma, Milan, Italy) and stored at - 20°C until use. Samples were further processed with the RNAEasy Mini Kit (Qiagen, Milan, Italy) for RNA extraction, following the manufacturer's instructions. The correspondance between cytologc and histologic findings in terms of B-RAF V600E is well known (32).

The diagnosis was defined 34 times preoperatively, in 107 cases was incidental.

PTMC was defined as incidental when it was found on the surgical histology samples from patients who had thyroid surgery for non-malignant thyroid disorders; it was described as nonincidental when the tumor was diagnosed preoperatively on the basis of fine-needle aspiration biopsy. In our series, no

PTMC were diagnosed by means of cervical lymph-node or distant metastases. In mcrocarcinomas incidentally diagnosed, the indications for thyroidectomy were compressive symptoms (46 patients) or toxic goiter (27 patients).

Total thyroidectomy was defined as total bilateral extracapsular thyroidectomy.

When the diagnosis was estabished preoperatively or strongly suspected intraoperatively, because of suspect thyroid nodule together with enlarged lymph nodes in the VI level and/or in lateral compartment were found, a systematic compartment-oriented lymph-node dissection was performed. Central compartment lymph-node dissection is defined by complete level VI dissection, and lateral neck lymph-node dissection is a level II to level V dissection.

Tumors were defined as multifocal if two or more tumor foci were found in one (unilateral) or both (bilateral) thyroid lobes, and the largest tumor dimension was used for statistical analysis.

All patients in whom a PTMC was found in histological sample, were submitted to postoperative (2-4 months) ablation of thyroid

residual.

According to literature (18), the criteria for successful 131I thyroid residual postoperative ablation were defined as the disappearance of any visible radioiodine uptake in the thyroid bed, and a overall radioiodine neck uptake <1%, and undetectable serum thyroglobulin off levothyroxine after obtaining a serum thyrotropin level (TSH) ≥30 IU/ml.

All patients in whom a diagnosis of papillary thyroid microcarcinoma had been previously made were considered for this study. Follow-up evaluation was obtained by outpatient consultation and/or telephone interview. 96 patients accepted the interwiev and were enrolled in the trial. On the contrary 45 patients were non found, or refused the clinical study. In patients enrolled age, sex, type of diagnosis, (incidental or nonincidental), autoimmune thyroid disorders, (Graves' disease, Hashimoto's thyroiditis), type of lymph-node dissection, thyroid volume, tumor size, tumor multifocality, extrathyroidal tumor extension and tumor–node–metastases (TNM) staging were investigated.

As the B-RAFV600E was available since 2006, the patients were divided in two groups: 2003-2005 (34 patients), in wich prognostic factors such as age, tumor size, uni/multifocal disease, were evaluated; and 2006-2010 (62 patients) in wich search of B-Raf mutation in tumoral tissue were evaluated together with the previous. We evaluated the association between these indicators and aggressiveness indicators such as extracapsular spread, metastatic lymph nodes (if a central neck dissection was performed) and tumour recurrence, defined as immediate or late 131I residual ablation.

The data were collected in currently available data base (Microsoft Excel® for Mac, version 12.1.0 - 080409).

Results

Of 96 patients, 76% were females and 22,9% men; their medium age was 50,4.

The recurrencies rate was 8,3% (8 patients). The cancer was unifocal in 85 patients (88,5%) and bifocal in 11 patients

(11,5%); the capsule invasion in the unifocals was 86,5% (83 patients) and in the bifocals 12,5% (12 patients).

9 patients (9,4%) had lymph node positive for cancer and 18 (18,8%) negative; in 69 patients (71,9%) the lymph node have not been evaluated.

The BRAF-V600E mutation was detected in 33 patients; of these in 26 (27,1%) the mutation was absent and in 7 (7,3%) was present; the BRAF mutation was not evalueted in 65 patients (65,6%) (Tab. 2).

There were no recurrences in the cancer < 3 mm but 2 (3,8%) in the cancers between 5-6 mm; 3 (11,5%) in the cancers between 6-7 mm and 4 (36,4%) in those > 7 mm. The p-Value, calculated with Chi-square, was < 0,001. The recurrences in the patients with capsular invasion were in 2/83 (2,4%) of unifocals and in 6/12 (50%) of bifocals. The p-Value, calculated with Fisher's exact test, was < 0,001. The recurrences in the unifocals cancers were 6/85 patients (7,1%) and 2/11 patients (18,2%) in the multifocals. The p-Value was 0,23.

The recurrences in the patients with lymph nodes positives were

3/9 (33,3%) and 4/18 (22,2%) in the patients with lymph nodes negatives with p-Value < 0.001.

The BRAF was in 3/7 recurrences (42,9%) with p-Value < 0.01.

The BRAF was present in 3/7 (42,9%) patients. The tests used for statistical analys of recurrences in the last two cases was the Fisher's exact test (Tab.3).

Table 2: General characteristics of 96 patients in the study

	N	**%**
Patients, total (%)	96	(100)
Sex, n (%)*		
- **Females**	73	(76)
- **Males**	23	(22,9)
Age, mean (±SD)	50,4(± 12)	
Year diagnosis, n (%)		
- **From 2003 to 2005**	34	(35,4)
- **From 2006 to 2010**	62	(64.6)
Tumor size, mean in mm (SD)	5,6 (± 2,3)	
Recurrence, n (%)		
- **Yes**	8	(8,3)
- **No**	88	(91,7)
Extra-capsular spread, n (%)		
- **No**	84	(87,5)
- **Yes**	12	(12,5)
Foci, n (%)		
- **Unifocal**	85	(88,5)
- **Multifocal**	11	(11,5)

Lymph nodes, n (%)		
- Not evaluated	69	(71,9)
- Lymphoadenectomy with L-	18	(18,7)
- Lymphoadenectomy with L+	9	(9,4)
B-RAF, n (%)		
- Not performed	63	(65,6)
- Abesent	26	(27,1)
- Present	7	(7,3)

Table 3: Risk factors for cancer recurrence

	Presence of recurrence* n/N (%)	**P-value**
Sex		
- Females	5/73 (6.8)	0.43[a]
- Males	3/23 (13)	
Age		
- 0 to 40	2/22 (9.1)	0.73[b]
- 41 to 50	2/26 (7.7)	
- 51 to 60	3/26 (11.5)	
- 61 or more	1/22 (4.5)	
Year diagnosis		
- From 2003 to 2005	5/34 (14.7)	0.34[a]
- From 2006 to 2010	3/62(48.3)	
Tumor size, in mm		
- 0 to 4	0/33 (0.0)	<0.001[b]
- 5 to 6	1/26 (38.8)	
- 6 to 7	3/26 (11.5)	
- >7	4/11 (36.4)	
Extracapsular spread		
		<0.001[a]
- No	2/84 (2.4)	
- Yes	6/12 (50.0)	

Foci		
- Unifocal	6/85 (7.1)	0.23[a]
- Multifocal	2/11 (18.2)	
Lymph nodes		
- Not evaluated	1/69 (1.4)	<0.001[a]
- Lymphoadenectomy with L-	4/18 (22.2)	
- Lymphoadenectomy with L+	3/9 (33.3)	
B-RAF (n=96)		
- Not performed	5/63 (7.9)	<0.01[a]
- Abesent	0/26 (0.0)	
- Present	3/7 (42.9)	
B-RAF (n=33)		
- Absent	0/26 (0.0)	<0.01[a]
- Present	3/7 (42.9)	

[a] Fisher's exact test, two-sided
[b] Chi-square for trend

Discussion

Thyroid cancer is one of the most important malignant tumors of the endocrine system. Its incidence is increasing over the years, approximately 1% of all the new diagnoses of cancer. Its etyology appears to be multifactorial, being due to the interaction between environmental factors, among which the most important are exposure to radiation and the lack of iodine in the diet, and genetic factors.

The expansion of knowledge about genetic mutations occurrinng in different thyroid tumors has characterized recent years, allowing the identification of a correlation between

specific mutations and phenotypic characteristics of thyroid cancers.

An example is represented by BRAF mutation that appears to be an indicator of aggressive behavior of PTC. Studies of this mutation were later exteded to the development of new targeted terapie for thyroid cancer such as the ones represented by inhibitors of RET and BRAF-dependent tyrosine kinase activity.

The results of these trials should provide us with the therapeutic efficacy of these treatments and their potential use, whether developed as monotherapy or as associations of multiple drugs, especially in the treatments of aggressiv thyroid tumors.

The application of genetic research of particular mutations to the diagnosis has allowed to improve the cytological diagnosis in those samples with FNA cytology indeterminate and/or atipic. The identification of BRAF mutation is particularly promising since the simple realization and the high specificity of the analysis for the determining of malignancy (2).

Since B-RAF mutation had been first descrive (34) its role in

tumorogenesis of papillary thyroid carcinoma has been well studied and the specificity of this mutation for PTCs has been pointed out. BRAF mutations are common in thyroid cancer as well as in melanomas: this fact can be explained with the role of cAMP in cell growth of both melanocytes and thyreocytes. In these cell types, cAMP activates MEK1 and extracellular signal-regulated kinases through mechanisms that may differ but that converge on BRAF. The role of MAPK pathway in pathogenesis of PTC has been well studied elsewere (29,35 ,36) first underlined the strong association between BRAF mutation and poor prognosis of PTC. A few time after (28) has been confirmed that BRAF mutation is specific for cancer arising from papillary line, such as classic variant, poorly differentiated or anaplastic thyroid carcinomas. Since the potential application of mutational analysis of BRAF in thyroid FNAB has been well documented (37). Several studies emphasized that BRAF mutation detection in FNAB specimens can help diagnose and identify those patients who may need more aggressive surgical treatment and vigilant clinical monitoring (32,38,39) .

It is important to underline that all studies showed the

association between BRAF mutation and increased recurrence rate (40) and even mortality (41). Multivariate analyses showed that the prognostic power of BRAF mutation does not depend from classical clinicopathological prognostic factors, such as age, capsular invasion, multifocality, lymph node methastases (40, 42). These data leaded to investigate the role search of BRAF mutation in optimizing the risk management of papillary thyroid microcarcinoma, as well as PTCs > 1 cm. Several studies, of which two italian (43, 44, 45,) demonstrate the association between BRAF mutation and clinicopathological aggressiveness of PMTC. However (45) analysis of differential BRAF(V600E) mutational status in high aggressive papillary thyroid microcarcinoma stated the difficulty to find any case of ‘‘clinical’’ recurrence of PTMC over short follow-up periods, so the predictive role of BRAF mutation for the recurrence of an indolent tumour such as PTMC could not be directly established. At the moment it is unclear how the ‘‘clinical’’ recurrence could be defined. A short time of follow-up might be one reason for the lack of any recurrence.

Another explanation for the lack of recurrence might be that the

sensitive serum thyroglobulin testing and radioiodine body scan, which are worldwide routinely used for the detection of thyroid cancer persistence/recurrence were apparently not used in this study for the follow-up of PTC. Nevertheless, the highly significant association of BRAF mutation with the classical high-risk clinicopathological factors in PTMC demonstrated by the study again strongly suggests that, as in conventional PTC, BRAF mutation is also a predictor for the aggressiveness of PTMC and may therefore indicate a poor prognosis. Although further studies on PTMC are needed to directly test this hypothesis, the previous demonstration that BRAF mutation predicted PTC recurrence in patients with TNM stage I and II diseases (then, a group including several PTMCs) could be useful to support it (23,42).

As PTMC is associated with an extremely low mortality rate, the core of the clinical effort in managing this cancer is to prevent, identify, and manage its recurrence. Preoperative information of BRAF mutation status obtained through analysis of ultrasonography-guided fine-needle biopsy specimens of PTMC could be very useful in guiding the management of this cancer at

various stages, helping determine the extent of surgery, the need for radioiodine ablation, and the subsequent follow-up. In recent years we have seen a rapid rise in the incidence of thyroid cancer worldwide, with a very high increase in Sicily, considered at the moment one of most stricked area in the world (21). It is unclear if the reason of this increase is an improved diagnosis or a real exasperation of the tumour (46, 47). Given that PTC accounts for 80% of all thyroid cancers and that nearly 50% of the new cases of PTC are PTMC, (46,48) a major challenge in managing thyroid cancer is how to appropriately manage the already large and still increasing number of PTMC cases. Although the overall prevalence of BRAF mutation in PTC is relatively high, around 45% on average,(49) the prevalence of this mutation in PTMC is generally much lower in many parts of the world (50, 51,52, 53) and as low as 18% in PTMC of 5 mm in size (53). Usually, the prevalence of BRAF mutation in PTC with TNM stages I and II, which consist largely of small tumors, is around 30% (49). With the relatively low prevalence of BRAF mutation in PTMC, it seems feasible to treat more aggressively the one-third of PTMC patients that

have BRAF mutation and may be thus prone to a poor prognosis. The remaining cases (majority) of the PTMC patients that are BRAF mutation-negative could be relatively conservatively managed unless indicated otherwise by other factors. The current risk management of PTMC is virtually exclusively based on clinicopathological criteria. By adding BRAF mutation as a new dimension in risk stratification of PTMC, the appropriate extent of surgical and medical treatments of this cancer may now be better determined.

Our study confirmed the association between well-known risk factors, such as multifocality or lymph node involvement and recurrence of disease. The limited number of thyroid multifocal microcarcinoma not allowed us to find a clear association between this specific risk factor and disease relapse. On the contrary, the size of tumour seems to be strictly correlated to this risk: infact, we had never obsesved a relapse for tumors < 5 mm, a low incidence for tumors between 5 and 6 mm of diameter (3.8%) and a progressive increase of risk as far as tumors > 7 mm, that showed a recurrence in 4/11 cases (36,4%).

An important association has been found between B-Raf mutation and risk of recurrence: in 3/7 patients presenting the mutation a relapse was observed, while none of 26 patients having a B-Raf wild type expressed the recurrence. It should rather be underlined the low incidence of B-Raf mutation in the total amount of thyroid microcarcinomas (only 7 patient out of 33 sampling showed the mutation). On the other hand it should be acknowledged that thyroid microcarcinoma is largely diagnosed incidentally. As a consequence, it is not usual to perform a fine-needle aspiration biopsy in the presence of an infracentimetric thyroid nodule, then the search of B-Raf mutation in thyroid nodules < 10 mm cannot be considered a routinary practice. In spite of that, we aim to perform fine-needle aspiration biopsies in the presence of thyroid nodules having suspected pattern, such as irregular edges, intralesional microcalcifications and/or vascularization, and a diameter of 5 mm at least. This behavior led us to two major evident results: an improved possibility to obtain a correct preoperative diagnosis; a precise prognostic statement.

The impact of medical and surgical interventions on the survival

of patients with PTMC is a topic widely discussed and there are many controversies about the management (1).

According to the authors overall actuarial survival rates, at 10 and 15 years, are 96.6% and 96.3%, respectively. In their experience increasing age was the only statistically important predictor for disease-specific survival ($p = 0.001$). Neither the type of surgery (total thyroidectomy, near-total/subtotal thyroidectomy or lobectomy), nor the radiometabolic treatment has any impact on excellent prognosis of patients with PTMC. The Authors conclude, that patients lacking evidence of metastatic disease may undergo lobectomy alone (54). The concept of limited surgery is supported also by many other Authors (54,55, 56). Bilimoria et al.(56) analysed almost 40,000 patients from the National Cancer Data Base with papillary thyroid carcinoma and concluded that the treatment of choice for patients with a tumour less than 1 cm is thyroid lobectomy (56).

Some Authors are of the opinion, that surgery, even if limited, may not be the first step of treatment (12). Ito et al. (57) selected low-risk patients with PTMC and, during 5 years'follow-up, only 6.7% of these patients were confirmed as showing an

increase in size at US compared to baseline findings. None developed additional distant metastasis or died of thyroid carcinoma. The Authors conclude that surgical treatment after the appearance of carcinoma progression is not late (57).

On the other hand, PTMC is often multifocal; the range of multiple non-contiguous tumour foci is between 18 and 87%, depending upon the technique used for the pathological analysis (102). The genetic studies, which compared the specific patterns of monoclonal X-chromosome inactivation (58) and distribution of BRAF mutation (59) demonstrated that the individual tumour foci often arise as independent tumours and, indeed, that they can grow and spread.

Fundamental for future clinical purposes is to determine clinical and especially molecular parameters which would define small groups of PTMC with aggressive biological behaviour. The most promising genetic is determination preoperative of BRAF mutation.

It could help to determine the choice of therapeutic management of this cancer and to assess the extent of initial surgical treatment and the need for radioiodine ablation (7).

In oncology, since alterations of BRAF are widely represented in many solid tumos it is the considerable interest directed to the development of specific inhibitors of BRAF activity (28).

Among these , the best known is the biaryl urea BAY 43-9006 (sorafenib) (60); it is an inhibitor active against RAF multikinase and other proteinkinase (VEGGFR-2and PDGRF), with can effectively block the kinase activity of BRAF (24, 61).The BAY 43-9006 has been tested on several types of human carcinomas including the thyroid cancer, and preliminary results showed a minimal or partial responce in some patients (62).

Conclusion

The present study allowed us to reach the following conclusions:

1)We corroborate the reliability of "classic" risk factors for the papillary thyroid carcinoma, expecially extracapsular spread and lymph node involvement, that showed to be significantly associated to a poor prognosis. also in microcarcinomas;

2)We esteem that it should be better fixed the limit of size

defining a low risk class of papillary thyroid carcinoma: infact, we observed that a diameter > 7 mm is related to a prognosis similar to papillary thyroid carcinomas > 1 cm. So, it seems that the risk stratification in the category of "microcarcinoma" is continuously growing with a tendence to reach the risk of larger tumors.

3)The search of B-RAF V600E mutation in samples of FNAB of thyroid nodules ≤ 1 cm showed to be reliable in terms of diagnostic power and prognostic significance. Concerning the first item, we would underline that it has been proved, in our study as well as in the current scientific literature the improvement of diagnonstic sensitivity and specificity of the evaluation of B-RAF V600E mutation to identify malignancies in thyroid nodules, expecially if ≥ 5 mm.

We would clarify that the meaning of "recurrence" in our experience is not a real progression of disease. Concerning a tumor having an indolent behavior, such as papillary thyroid carcinoma, the recurrence should be considered a biochemical-imaging evidence of residual-recurrent thyroid tissue after

thyroidectomy for malignancy. In spite of that, this tissue is sensitive to usual tratments, such as radioiodine, and after a new radiometabolic treatment the thyroglobulin as well as radionuclide fixation of thyroid bed usually shows an improvement and even a disappearance of disease signs.

The finding of this mutation in samples of thyroid FNAB is associated to a poor prognosis. It could lead to more aggressive attitudes in terms of diagnosis, treatment and postoperative immediate and late controls.

A longer follow up is needed to confirm these findings and to identify a cathegory of patients involved in a real recurrence of disease.

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