Heterogeneity of Thyroid Cancer

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Keywords

Thyroid cancer · Heterogeneity · BRAF mutation · RAS mutation

Abstract

There are 5 main histological types of thyroid cancers (TCs): papillary, follicular (also known as differentiated), poorly differentiated, anaplastic (the most aggressive form), and medullary TC, and only the latter arises from thyroid C cells. These different forms of TCs show significant variability, both among and within tumours. This great variation is particularly notable among the first 4 types, which all originate from thyroid follicular cells. Importantly, this heterogeneity is not limited to histopathological diversity only but is also manifested as variation in several genetic and/or epigenetic alterations, the numbers of interactions between the tumour and surrounding microenvironment, and interpatient differences, for example. All these factors contribute to the great complexity in the development of a tumour from cancer cells. In the present review, we summarise the knowledge accumulated about the heterogeneity of TCs. Further research in this direction should help to gain a better understanding of the underlying mechanisms contributing to the development and diversity of TCs, paving the way toward more effective treatment strategies.

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Introduction

Thyroid cancer (TC) is the most common malignancy of the endocrine system, accounting for 3.4% of all cancers diagnosed annually worldwide. Accumulating evidence of the rapidly growing number of TC diagnoses have led to a discussion of whether or not this disease currently represents an epidemic, which may be related to different causes [1]. The most important factor contributing to the increased TC diagnosis rate is better detection tools, including the widespread application of thyroid ultrasound and an increase in the number of thyroid biopsies performed. On the one hand, this overdiagnosis phenomenon probably prevails over the real changes in TC epidemiology associated with an increasing life span, variability in iodine intake, and potential exposure to radiation in isolated populations. On the other hand, novel emerging therapies have focused attention on the previously neglected population of patients with advanced TC, which is often incurable and associated with highly symptomatic presentation in some cases.

The broad heterogeneity of TCs represents one of the most spectacular examples of variability amongst all malignancies. TCs range from a thyroid papillary microcarcinoma (according to the definition of a malignant disease, although its incidence is reportedly up to 30%

among healthy people) to anaplastic TC (ATC), one of the deadliest malignancies, which is fatal in the vast majority of cases even if radically resected at an early stage. In certain populations, TCs, although clearly malignant, might only occasionally lead to death, including cases of papillary TC (PTC) in children and young adults presenting with lymph node metastases (LNMs). Some TC subtypes were recently reclassified from malignant to benign, such as non-invasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP). Although a number of excellent studies have been carried out to elucidate the heterogeneity of traditionally common cancers such as melanoma and breast, lung, colon, prostate, and renal cancers [2], TC has rarely been addressed in this context.

Origin and Frequency

According to the World Health Organization classification, there are 5 main histological TC types: PTC, follicular TC (FTC), poorly differentiated TC (PDTC), medullary TC (MTC), ATC. PTC and FTC are considered to be differentiated TCs (DTCs), which represent the vast majority of all TC cases at 80 and 10%, respectively. Through dedifferentiation, DTCs may lead to the more poorly differentiated and lethal undifferentiated forms of TC. MTC arises from C cells and, despite evolving in the same stromal microenvironment, demonstrates a clinical course and molecular basis that are completely distinct from those of non-MTCs.

Prognosis

DTCs represent rather indolent tumours characterised by a good response to standard treatment with a 10-year survival rate reaching 90% [3]. Nevertheless, approximately 10% of all DTC patients die of the disease. Moreover, recurrences are now more frequently reported [4–6]. PDTC and ATC are associated with a poor prognosis, and the majority of these tumours are refractory to radioiodine.

Based on the current knowledge, it is assumed that intratumour heterogeneity develops through certain genetic and/or epigenetic changes in tumour cells, due to the influence of the tumour microenvironment, or as a consequence of interactions among all of the above-mentioned factors [7–9]. The formation of diverse cell subpopulations reflects the response of a tumour to changes within the microenvironment. Moreover, this process enables the creation of more aggressive subpopulations that can spread throughout the body and form secondary tumours or acquire resistance to specific therapeutic agents. The variability in tumour cells may be a consequence of

genetic factors, non-genetic factors, or interactions between them. Genetic diversity within a single tumour is believed to result from multiple events, including overcoming the telomere crisis, acquiring genetic mutations, and appearance of a driver mutation required for malignancy initiation [10, 11]. In turn, the non-genetic heterogeneity is mainly associated with epigenetic abnormalities; since some of these changes can be functionally similar to genetic mutations, they are referred to as epimutations. Aberrant methylation of the promoter regions of genes, which are responsible for cell cycle regulation, may increase genomic instability. Such epigenetic instability may also be related to the remodelling of chromatin as a consequence of post-translational modifications of histone proteins and to some genetic disorders. Moreover, the potential role of abnormalities in microRNA expression due to epigenetic changes cannot be overlooked, which may ultimately impact gene expression.

The tumour microenvironment, composed mainly of inflammatory cells, fibroblasts, and extracellular matrix, is the major source of selection pressure and the key external factor that impacts the phenotypic diversity of tumour cells. The relationship between the tumour cells and stroma, the so-called “heterotypic interaction,” is an integral part of phenotypic plasticity of tumours, which is also a consequence of autocrine and paracrine mechanisms related to both tumour and normal cells [12]. Such phenotypic plasticity may also be exhibited by genetically identical cells, representing the so-called “stochastic heterogeneity” phenomenon; the most-well-studied cause of this heterogeneity is gene expression changes that affect biochemical processes. The stochasticity of gene expression may be involved in a transition between distinct epigenetic or differentiation states related to phenotypes that have a significant impact on the clinical course [10].

With this review, we sought to provide an overview of the phenomena representing the heterogeneity of TC considering its pathology and molecular biology. Much of this heterogeneity is represented by the different histopathological types and subtypes that display distinct molecular backgrounds, intratumoral dissimilarities, and clinical outcomes. Moreover, the interpatient heterogeneity and stochasticity of biochemical processes within the tumour add to this intrinsic variability, leading to a multiplicity of potential phenotypes. The tumour itself is a very dynamic formation of cells that progress, adapt to new microenvironmental conditions, and acquire resistance to medical therapies, thereby representing a substantial clinical challenge. Thus, it is imperative to gain a

Table 1. Levels of thyroid cancer (TC) heterogeneity

Level of heterogeneity	Example
<i>Intrapatient (intratumour)</i>	
Stochastic	Variability between cells, both at the level of morphology and molecular alterations (e.g., passenger mutations), not yet classified at higher levels
Phenotypic functional plasticity	Epithelial-mesenchymal transition
Epigenetic modifications	Downregulated expression of tumour suppressor genes caused by DNA methylation (FTC)
Nuclear heterogeneity	Papillary-like carcinoma features: grooves, inclusions, optically clear nuclei Convolutated nuclei (PDTC)
Cytoplasmic heterogeneity	Hobnail features (PTC), oncocytic features (FTC) Rhabdoid morphology (ATC)
Molecular clonality	Multifocal PTC, primary tumour vs. distant metastases
Degree of differentiation	Mitotic activity/necrosis in PTC/FTC
Stroma	Desmoplasia, psammoma bodies (PTC), amyloid (MTC)
Other cells present or infiltrating the tumour and its stroma	Dendritic cells, macrophages, lymphocytes, mast cells, fibroblasts
Tumour compartment/ functional area	Invasion front vs. central compartment Minimally invasive vs. widely invasive variant of FTC
Morphological variants/ architecture	Presence of capsule (PTC, FTC) Classical variant of PTC and TCV Insular pattern in FTC
Tumour focality	Multifocality in PTC
Primary focus vs. metastatic site	Distant metastases of PTC: enrichment in <i>TERT</i> mutations and a decrease in <i>BRAF</i> mutations (see Melo et al. [110])
Histotype	PTC, FTC, PDTC, ATC, MTC
Cell of origin	Follicular vs. parafollicular differentiation (rarely observed mixture of lineages, e.g., mixed medullary-follicular thyroid carcinoma [105])
<i>Interpatient (intertumour)</i>	
Molecular and functional tumour heterogeneity	Different extent of tumour variability (heterogeneity in various patients as described above)
Morphological variants/ degree of differentiation	In PTC: classical variant, TCV, CCV, HV, SV In FTC: necrosis, mitotic activity
Histotype	PTC, FTC, PDTC, ATC, MTC
Cell of origin	Follicular origin, parafollicular origin
Patient-related factors	Age, sex, genetic background, family cancer history, other diseases

ATC, anaplastic TC; CCV, columnar-cell variant; FTC, follicular TC; HV, hobnail variant; MTC, medullary TC; PDTC, poorly differentiated TC; PTC, papillary TC; SV, solid variant; TCV, tall-cell variant.

better understanding of these challenges so as to find effective strategies to overcome them. However, ongoing research in this field continues to highlight the complexity of thyroid tumours.

Levels of Heterogeneity in Thyroid Malignancies

Although originating from the same cell type, the non-MTCs represent a very heterogeneous group of tumours with significant differences regarding histopathology, biological behaviour, oncogenic events, and clinical course.

This broad TC diversity does not only derive from the particular type or histopathological subtype but is also due to interpatient variability and intratumour heterogeneity (Table 1). This situation represents a particular challenge for personalised cancer medicine, since cancer treatment is currently based mainly on the diagnosis of a sample obtained during surgical excision. Regarding follicular neoplasms, the final diagnosis is achieved based on surgically resected tumours. Such material can only provide information about a small region of the tumour and thus may not be representative of the variation within a tumour as a whole.

Pathological Assessment of Heterogeneity

The heterogeneity of thyroid neoplasms is routinely characterised pathologically by examining the primary thyroid tumour and metastases at both the macroscopic and microscopic level.

Intratumour heterogeneity depends on the tumour invasion front (cells able to invade the tumour capsule and stroma) in opposition to the central tumour compartment (cells of unknown invasiveness). In TC, the central compartment rarely displays necrotic features due to the high growth kinetics or nutrient supply, which are also common features of high-grade tumours of other organs. This type of necrosis is only noted in PDTC and ATC, and very rarely in MTC. It is important to make the distinction between central tumour cell necrosis and that of other areas, such as the necrotic features of oxyphilic tumours. In some cases, extensive tumour necrosis spares surrounding blood vessels, producing peritheliomatous appearance [13]. On the contrary, necrosis noticed after fine-needle aspiration biopsy in oncocytic thyroid tumours is usually coagulative and extensive. An extensive sampling of the infarcted lesion may show a focal residual viable tumour at the periphery. The infarct may be accompanied by tissue repair [14]. Cellular heterogeneity affects both tumour and stromal cells. Tumour cells may exhibit follicular differentiation, parafoollicular differentiation, or both types of differentiation or dedifferentiation. The various stromal changes observed in TC include those of the psammoma bodies, fibroblasts, endothelial cells, and tumour-associated inflammatory cells. Among the TC subtypes, PTC tends to show the highest heterogeneity of the stroma. For example, the degree of desmoplastic stromal reaction (desmoplasia), which is the presence of a newly formed collagenous stroma surrounding the invasive epithelial tumour cells [15], has been suggested to be associated with the clinical outcome of PTC (discussed in more detail below) and is a reliable and highly reproducible predictive parameter of the metastatic potential of MTC [15].

Models of Thyroid Carcinogenesis and TC Heterogeneity

Cancer Clonal Evolution

There are 2 major hypotheses put forward to explain the processes of tumour formation and its heterogeneity: the classical (stochastic) multistep carcinogenesis model, which is based on a theory of cancer clonal origin, and the most recent hypothesis based on cancer stem cells [16]. The multistep model states that tumour formation is a

Fig. 1. Simplified scheme of a multistep theory of thyroid carcinogenesis.

consequence of genome instability within somatic cells and the appearance of more aggressive clones that survive the selection pressure of the microenvironment. The appearance of a functionally significant mutation leads to divergence of a new subclone, which can dominate and outcompete other cells to result in a homogenous tumour until a new, significant, and more versatile, mutation appears [11]. According to this theory, FTC is considered to be derived from follicular adenoma, whereas PTCs originate from thyroidocytes after mutations in oncogenic genes, including the *BRAF* V600E mutation or rearranged during transfection (*RET*) gene rearrangements (Fig. 1) [17–19]. However, one should be aware, despite that some benign lesions can evolve, defining adenoma as a “pre-malignant” lesion is not justified.

PDTC and undifferentiated TCs may develop from both DTC types after acquiring further alterations in phosphatidylinositol-3-kinase (*PI3K*) and *WNT* pathway genes or in suppressor genes (mainly TP53 mutations) [20]. On the one hand, the multistep theory could explain the accumulation of multiple insults leading to an ATC genomic profile. On the other hand, thyroidocytes in adults rarely proliferate, and thus there is a low probability of acquiring PDTC features through accumulated mutations. The other doubts related to the multistep theory in TC are related to the fact that genetic alterations that are characteristic of differentiated thyroid tumours, such as *RET*/*PTC* and *PAX8*/*PPRγ* gene rearrangements, are not present in ATC [21, 22], as well as *NTRK* rearrangements, which are rarely observed in ATC [23].

Stem Cell Model

The second hypothesis, the TC stem cell model, assumes that there is a small subpopulation of stem cells after genetic and epigenetic transformations that could produce phenotypically different cancer cells. The first paper related to TC stem cells was published in 2007 [24].

Fig. 2. Simplified scheme of the cancer stem cell/foetal theory of thyroid carcinogenesis.

The most complex model regarding cancer stem cells in the thyroid was proposed by Takano [16] as the foetal thyroid carcinogenesis theory. This model states that thyrocyte stem cells after genetic/epigenetic changes generate ATC, and non-transforming thyroid stem cells generate a multipotent progenitor cell type termed thyroblasts (Fig. 2), which can readily develop into PTC or FTC, while benign tumours are formed from the thyrocyte precursor cells (prothyrocytes).

Phenotypic and Molecular Heterogeneity of TC Subtypes

Papillary Thyroid Carcinoma

Most PTC tumours present irregular borders and an infiltrating thyroid parenchyma. Well-defined tumours can be surrounded by a capsule, as observed in NIFTPs, which are considered to be benign tumours.

Heterogeneity within a given histological type can present among multiple foci in the thyroid, including multiple PTC. Nevertheless, such heterogeneity detected at the microscopic level does not necessarily reflect the degree of heterogeneity present at the molecular level. Indeed, several studies have confirmed the high intratumour variation in the PTC dissimilarity of each single tumour [25, 26].

PTC rarely exists as a microscopically homogenous tumour (for example only the classical variant). The classical variant is often accompanied by the follicular variant. The coexistence of 2 histological PTC variants can be clinically relevant if one of the variants represents a more aggressive type such as the tall-cell variant (TCV), columnar-cell variant (CCV), hobnail variant (HV), and solid variant. Only 10% of PTCs with indication of the presence of an aggressive variant are reported to involve the

TCV as the dominant variant. However, recurrences and metastases in such cases can be associated with a higher TCV percentage [27]. There is no consensus in the literature with regard to the minimal area of tall-cell morphology required for a TCV diagnosis, with a range of 10–70% TCV cells reported [28]. Currently, TCV in PTC is considered when at least 50% of the cells show the characteristic TCV morphology [27].

A case of TCV-PTC with Hurthle cell carcinoma was described by Baloch et al. [29], who demonstrated 2 distinct patterns of metastases: the TCV metastasised to regional lymph nodes, whereas the Hurthle cell carcinoma presented with pulmonary metastasis.

The CCV of PTC is typically diagnosed in tumours in which at least 30% of the cells are columnar cells [30]. Tumours with a CCV component that are encapsulated or well circumscribed without extrathyroidal extension were shown to display relatively more indolent behaviours [31].

The HV of PTC is another aggressive variant that can be found in heterogeneous cases of PTC along with tall-cell and anaplastic carcinoma [32]. A report of case series demonstrated that the HV-PTC form typically represents >30% of the tumour [30]. More than half of all reported patients with HV-PTC have distant brain, bone, liver, and lung metastases. The hobnail features of PTC emerged as parameters that were strongly associated with LNM risk in a study conducted by Chung et al. [33]. Tumours are classified as PTCs with hobnail features if they reveal apically localised cuboidal-to-oval cells that produce a luminal formation in >5% of a given tumour section. These heterogeneous tumours more frequently present with LNMs. Similarly, the presence of micropapillary structures <200 µm in size and the absence of fibrovascular structures in >20% of the tumour section can indicate a higher risk of LNMs.

The majority of PTCs are characterised by the presence of >1 anatomically separated focus, which has been described in 18–87% of reported PTC cases [34–38]. However, there is still a lack of consensus on whether this occurrence is a consequence of multiple independent tumours or due to the intrathyroidal spread from a primary single tumour mass. Both hypotheses, as presented by Kuhn et al. [39], were confirmed in several studies. Despite the differences in molecular techniques among these studies, the common conclusion was that PTC multiplicity could be a result of either multicentricity or intrathyroidal spread [40–42]. One particularly interesting finding was that the ipsilateral PTC foci showed the same X chromosome inactivation pattern, and discordance was observed between contralateral nodules [39]. Moreover, McCarthy et al. [43] provided molecular evidence that separate coexisting papillary thyroid tumours arose from the same clone, pointing to intrathyroidal metastasis as a likely cause of multifocality. However, there was no information provided as to whether or not the nodules were from the same or contralateral lobe.

As mentioned above, PTC exhibits marked heterogeneity with respect to its stromal cellular composition and architecture. Stromal reactions in PTCs include desmoplasia, nodular fascitis-like changes, fibromatosis-like changes, inflammatory myofibroblastic tumour-like features, myxoid areas, elastosis, and amyloid formation [44]. Desmoplasia seems to be an indicator of the invasive behaviour of papillary thyroid microcarcinomas, and it is significantly associated with LNMs [45]. The expression of hypoxia-inducible factor-1 α has also been associated with desmoplasia within the tumour and thus may play an important role in the invasive behaviour of these tumours independent of the *BRAF* mutation status [46].

One of the historically defining features of PTC is the presence of psammoma bodies, which also show significant heterogeneity in their occurrence. Psammoma bodies are defined as spherical, calcified foci with concentric laminations located in the stroma [47]. Bai et al. [48] confirmed that the presence of PTCs with psammoma bodies was associated with poorer disease-free survival compared to PTC without psammoma bodies. Moreover, they observed upregulation of bone morphogenetic protein 1 expression in carcinomas with psammoma bodies, and Carcangiu et al. [36] described a higher incidence of nodal and pulmonary metastases in PTCs containing psammoma bodies.

The heterogeneity of immune cells in the tumour stroma also plays an important role in clinical outcome. The density of mast cells was found to be higher in 95% of

PTCs compared to that in control normal thyroids, and the extent of mast cell infiltration in PTCs was correlated with capsule invasion, extrathyroidal extension, and a poor prognosis [49].

Some researchers have searched for differences in the mutational status between particular nodules in the *RET* [50] or *BRAF* [51] genes, which are the most frequently altered genes in PTC and can initiate PTC development, as demonstrated with mouse model studies [52, 53]. However, these alterations may also occur later during the carcinogenesis process, based on comparisons of the gene status between primary tumours and LNM or distant metastases. For instance, Vasko et al. [54] observed a discordance in the presence of the *BRAF* V600E mutation between primary PTC and LNMs. Although some patients with discordant forms displayed multiple PTC foci, the authors could not exclude the possibility that the lymph node-associated PTC tissues had not metastasised from these foci. Of note, there were also some cases without PTC multifocality. These findings suggested that a *BRAF* mutation may develop de novo in metastasised TC cells in the lymph nodes. Moreover, considering the results of Oler et al. [55], who found a deletion in the *BRAF* 601 codon only in the LNM and not in the primary PTC tumours, Vasko et al. [54] further suggested that the lymph node milieu may predispose the *BRAF* gene in TC cells to be more susceptible to alterations around codons 600 and 601, and might also create a favourable environment for the selection and survival of thyroid cells harbouring a *BRAF* mutation. In addition, Walts et al. [56] indicated that a discordance in the *BRAF* mutation status was rather infrequently detected within individual PTCs and between individual PTCs and the corresponding LNM at the time of thyroidectomy. However, the frequency of this discordance increased when the primary tumour and recurrent metastases were compared. Overall, 70–92.9% of reported cases showed concordance of *BRAF* mutation in the primary tumour and paired LNMs [55, 57]. However, only 33–40% of multifocal PTC cases showed a discordant *BRAF* status between foci [57]. To determine the phylogenetic relationships between matched primary thyroid tumours and metastases, Le Pennec et al. [58] performed paired-end massively parallel sequencing of 2 areas of the primary tumour, including 1 pleural metastasis (PLM) and 2 LNMs from a patient with aggressive PTC. Although all of the examined tissue samples were composed of several cellular subclones, the analysed areas of the primary tumour and one of the LNMs displayed similar genetic profiles, whereas the other LNM presented more divergent mutations and fusions.

Greater divergence was observed between the primary tumour and PLM. Thus, the ancestral mutations present in the primary PTC were propagated to the metastases, and additional genetic alterations were simultaneously found in the PLM. These data indicate that genetic alterations accumulate after clonal PLM separation from the primary clone. Although there is currently no clear answer as to whether cancer cells simultaneously spread from the primary thyroid tumour to the regional LNs and distant locations or first spread to the lymph node and then to further locations, Le Pennec et al. [58] suggested that the PLM originated from a subclone of the LNM.

With respect to intratumoral heterogeneity, the majority of studies have focused on the most prevalent genetic alterations in PTC, the *BRAF* V600E mutation and *RET* rearrangements. The polyclonality for *BRAF* within primary thyroid tumours was reported to be rather infrequent [59]. However, some analyses revealed contradicting results in which the occurrence of subclonal or even oligoclonal *BRAF* mutation was more frequent than the clonal presence [60, 61]. Guerra et al. [60] showed that in the majority of *BRAF*-positive PTCs, <50% of the cancer cells contained the *BRAF* V600E mutation, which suggested frequent *BRAF* heterogeneity. These contradictory data may result from different methods applied in the different studies: Abrosimov et al. [59] used direct sequencing, which could not reveal the heterogeneous presence of a *BRAF* mutation within a tumour, whereas Guerra et al. [60] used pyrosequencing, which is a more quantitative analysis that enabled such detection. However, a large Cancer Genome Atlas Research Network (TCGA) study, describing the genomic landscape of almost 500 PTCs, demonstrated that driver mutations in genes such as *BRAF*, *NRAS*, *HRAS*, *KRAS*, and *EIF1AX* were present in the majority of tumour cells and were largely clonal [62]. These results were consistent with other studies showing homogeneous cancer cell immunohistochemical staining with *BRAF* V600E-specific antibodies [63]. Studies on the second most prevalent genetic event in PTCs, *RET*/*PTC* rearrangements, have revealed that these alterations varied within individual PTCs and may be present in the majority, a small fraction, or only a few tumour cells [64, 65]. The possible explanations for this variation proposed by the authors included a multiclonal origin of the tumour and that *RET* rearrangements may constitute a later subclonal event. An interesting case was reported by Schopper et al. [26], who analysed a single thyroid tumour that showed a combination of conventional PTC, the follicular variant of PTC, clear-cell PTC, columnar-cell carcinoma, and PDTC. The authors used massively parallel

next-generation sequencing (NGS) using a laboratory-developed NGS panel of 8 cancer-related genes (*BRAF*, *KRAS*, *HRAS*, *NRAS*, *EGFR*, *PIK3CA*, *KIT*, and *PDGFRA*) to analyse each morphological component and metastasis. Surprisingly, no systemic underlying molecular connections were found between the analysed areas of the tumour, although all of the tested morphological areas were reported to have etiological or other associations with one another. Two of the detected morphologies, clear-cell PTC and PDTC, shared a common alteration in *BRAF*. *KRAS* mutations were detected within the primary tumour, but these differed between the CCV and follicular PTC variant. Conventional PTC was the only morphological type that was not associated with any mutation included in the panel. These data demonstrated significant discordance of the molecular signature within a single PTC tumour, highlighting its heterogeneous nature.

The aforementioned TCGA study [62] clearly demonstrated substantial intertumour heterogeneity in PTCs with respect to their molecular alterations. The studied PTC set consisted of almost 500 cases, which were analysed for their genetic, epigenetic, and proteomic background. By searching for somatic single-nucleotide variants, insertions, deletions, gene fusions, and somatic copy number alterations, the authors identified presumed cancer drivers in 98.8% of the PTC cases. Some of these have been reported to initiate PTC, whereas others were not previously described. The authors developed a new index termed the *BRAF* V600E-*RAS* score (BRS) to identify possible relationships across the samples in the cohort and their degree of similarity to a *BRAF*-like or *RAS*-like gene expression profile. The *BRAF*-like cohort consisted mainly of PTCs with the *BRAF* V600E mutation, which itself represented a very heterogeneous group. Moreover, the thyroid differentiation score was proposed to indicate the extent of divergence within *BRAF* V600E-positive PTCs. The significant gene expression variation detected within this set of samples may be related to the range of differentiation and could help to partly explain the doubts associated with using mutated *BRAF* as a single marker of poor prognosis. Large overall heterogeneity was also observed when unsupervised clustering was applied to 4 genomic data sets acquired within the TCGA study, in which different numbers of subtypes were identified in each of the sets: 5, 6, 4, and 4 subtypes were found when using mRNA expression, microRNA expression, DNA methylation, and protein expression data, respectively. In contrast to the *BRAF*-like PTCs, which could be divided into different numbers of subtypes according to the data type, ranging from 2 (DNA methylation data) to 5 (microRNA data),

the *RAS*-like PTC group formed a single cluster with all data sets, except for DNA methylation. The authors emphasised the significant differences between *BRAF*-like and *RAS*-like PTCs at the genomic, epigenetic, as well as proteomic levels, as well as the significant diversity among *BRAF* V600E-positive PTCs, emphasising the need to explore the molecular background underlying this variation in more detail. Mancikova et al. [66] also observed a divergence between *BRAF*- and *RAS*-positive PTCs in terms of epigenetic changes by examining the role of aberrant DNA methylation in the formation of well-differentiated thyroid tumours. They observed a dependency between specific methylation patterns and the presence of a FTC and PTC pattern and the underlying mutation type. Moreover, in comparison to PTCs, follicular tumours displayed a higher level of methylation. The DNA methylation patterns in follicular adenomas (FAs), which are considered to be an early stage of FTC, were slightly different from those observed in the normal thyroid, implicating a role in malignancy initiation.

NIFTP, although currently considered as a benign tumour [67], has been treated as TC until recently. The data regarding its molecular background are scarce and require further studies. This is mainly because the criteria for NIFTP diagnosis are still discussed. Current data indicate the presence of mainly *RAS* mutations, along with *PPAR γ* and *THADA* fusions [68]. However, some papers reported NIFTPs with the *BRAF* V600E mutation [69] or a coexistence of *TERT* promoter and *RAS* mutations [70]. Simultaneously, another paper emphasised the importance of tightening the classification criteria, since the *BRAF* V600E mutation has not been present in NIFTP cases, in which papillary structures were absent [69]. As demonstrated, NIFTPs may present indolent histopathological features, which do not correspond to the molecular profile rather observed in aggressive tumours. The above-mentioned data point to the necessity of further analyses to precisely determine the diagnostic criteria.

Follicular Thyroid Carcinoma

FTC is the second most common type of TC following PTC. Compared to PTCs, FTCs seem to be more aggressive, tend to be at a more advanced stage at the time of diagnosis, are less sensitive to traditional therapy, and more often cause mortality. The pre-operative diagnosis of FTC is very problematic because of its similarity to FAs, and the distinction between both lesions is currently based

mainly on the presence of capsular or vascular invasion. FTC and FA are not only similar with respect to histopathological features but also show a common genetic background [71]. *RAS* somatic mutations and *PAX8/PPAR γ* rearrangements, the key FTC alterations, were detected in both FTCs and FAs [72]. FTCs show relatively less phenotypic heterogeneity than PTCs; approximately 80% of FTC cases involve microfollicular or solid/trabecular growth, and well-formed colloid-containing follicles are found in approximately 20% of cases [73]. Only a small group of patients showing monotonous follicular tumours with capsular invasion only presented metastases, which could be explained by heterogeneity at molecular level [74]. A prominent spindle-cell component, or rhabdoid, oncocytic, or clear-cell phenotype, or signet-ring cells have been observed in rare FTC cases [75, 76]. Yamashita et al. [77] suggested that the insular component in FTC was an independent risk factor for distant metastases to the lungs or bones. Heterogeneity of the tumour border is an unusual finding during FTC follow-up. FTC usually has a well-formed capsule containing medium-calibre blood vessels, which can be thin (<1 mm) or thick (1–3 mm) [76]. Cases of widely invasive FTC tend to show an incomplete capsule; thus, capsular invasion could be an indicator of vascular invasion [78]. This is particularly relevant since the probability of aggressive behaviour increases in proportion to the extent of vascular invasion [73], and recurrences were found to occur in 50% of patients with encapsulated angio-invasive FTC [74]. Moreover, more extensive capsular invasion and FTC extension into the thyroid parenchyma are associated with higher metastatic and mortality rates [79].

Despite its more aggressive nature, the heterogeneity of FTCs has not been as extensively studied as that of PTCs. Apart from the above-mentioned genetic alterations, several other genes have been demonstrated to be mutated in FTC, albeit with a much lower frequency, including *PI3CA*, *PTEN*, *DICER1*, *EZH1*, and *SPOP* [80, 81]. Many of these genes are involved in the PI3K/PTEN/AKT pathway, which is believed to be the key signalling pathway in FTC development [82]. Da Silva et al. [83] analysed the intratumour heterogeneity of FTC at the molecular level based on a single FTC case with distinct morphological components, including follicular, macro-follicular/pseudopapillary, oncocytic, and poorly differentiated variants. The authors reported 2 chromosomal gains that were present in all compartments (11q and 17q), suggesting that these may be early events in tumour development, and demonstrated that chromosomal gains were more common than losses.

Poorly Differentiated and Anaplastic Carcinoma

Poorly differentiated carcinomas are characterised by an incomplete tumour capsule with expansive growth [84]. Microscopically, monomorphic carcinoma cells can create a heterogeneous structure along with insular, trabecular, and solid patterns in the majority of cases [13]. In a few cases, the main tumour may be accompanied by satellite nodules. When a well-differentiated carcinoma dedifferentiates, it may macroscopically resemble a colloid tumour (well-differentiated component), which is closely surrounded by a solid tumour with necrosis (poorly differentiated component) [13]. The probability of dedifferentiation of a well-differentiated carcinoma is low [85].

Clear contrast between the phenotypic heterogeneity of the tumour centre and tumour border was observed in a study of encapsulated thyroid tumours of follicular cell origin with high-grade features such as a high mitotic rate and tumour necrosis [86]. All 8 of the non-invasive tumours examined showed indolent behaviour despite the presence of focal/extensive tumour necrosis. The authors stressed that surgical removal of these non-invasive tumours with necrosis and high mitotic activity may have interrupted their biology and prevented their progression into high-grade invasive carcinomas.

PDTC is characterised by strongly reduced lymphocyte and dendritic cell infiltrates compared to PTC [87] and an increased number of tumour-associated macrophages (TAMs) [88]. Mutations in the *TP53* gene are the most frequent genetic alterations found in PDTC, which may reflect chromosome instability and TC progression [89]. These mutations seem to be a late event of thyroid carcinogenesis, which is in opposition to the stochastic model of thyroid tumorigenesis in which the *TP53* mutation is a crucial alteration of the progression of follicular tumours [23]. *RET/PTC* and *PAX8/PPRγ* rearrangements, characteristics of DTCs, are very rarely observed in PDTC, suggesting that these mutations are not related to cell dedifferentiation [90]. *RAS* mutations, which have also been described in PDTC, may be associated with metastatic capability [90, 91]. *BRAF* mutations are quite frequent in PDTC associated with papillary thyroid nuclear features or PTC foci.

ATC is the most heterogeneous of all the TC subtypes. ATCs are typically widely invasive tumours that extend into the thyroid parenchyma and to the surrounding soft tissue and structures of the neck. Grossly, ATCs are characterised by various features, which correspond to the heterogeneity observed at the microscopic level. Only the

paucicellular variant of ATC is monotonous at both the macroscopic and microscopic levels. ATC presents a broad spectrum of differentiation, consisting of an admixture of spindle cells, pleomorphic giant cells, and epithelioid cells [92]. Morphologically, ATC can simulate fibrosarcoma, undifferentiated pleomorphic sarcoma, solitary fibrous tumour, angiosarcoma, or rhabdomyosarcoma. Carda et al. [93] confirmed true skeletal muscle differentiation in 2 cases of ATC both immunohistochemically and ultrastructurally.

The stroma of ATC could be variably hyalinised, sclerotic, or desmoplastic [94]. A peculiar interconnected network created by ramified TAMs (RTAMs) is a characteristic feature of ATC, which was suggested to play a role in the crosstalk and molecular transfer between RTAMs and cancer cells, and to provide nutrition to the cancer cells from the blood [95].

Similar to encapsulated PDTCs, 3 cases of encapsulated ATCs have been reported, which were characterised by a good clinical outcome with an overall survival up to 57 months [96–98]. This suggests that a well-defined tumour capsule despite a morphologically anaplastic tumour centre can be an important prognostic factor in ATC. Thus, pathologists should report a more aggressive tumour type such as PDTC or ATC as the first diagnostic line, regardless of its size and proportion within the tumour nodule [94, 99].

ATC demonstrates a heterogeneous set of genetic alterations with a high prevalence of mitogen-activated protein kinase effectors [100] and genes involved in the PI3K and/or β -catenin signalling pathways. *RAS* and *BRAF* gene mutations are also highly frequent in ATC, reaching up to 50% [100]. *PIK3CA* copy number gain is another highly frequent molecular event characteristic of ATC, which often coexists with other somatic mutations such as *BRAF* mutations [101]. Interestingly, *PIK3CA* copy gains are also more frequent in FTC than *BRAF* mutations, which are the characteristic genetic alterations in PTC. *TP53* gene mutation is characteristic for ATC, with a prevalence of approximately 70% among ATC cases, but is not observed in differentiated thyroid tumours. It is suggested that this mutation is a late molecular event in thyroid carcinogenesis when considering the classical model of thyroid tumorigenesis. Alternatively, the detection of *TP53* mutations could be related to the presence of subclones representing a characteristic specific to only ATC development [23].

Medullary Thyroid Carcinoma

MTC is usually well defined and not encapsulated. In sporadic cases, MTCs are typically unilateral, whereas inherited tumours are characteristically multiple and bilateral. The heterogeneity of MTC mainly presents as dual differentiation of parafollicular and follicular cell lineages.

MTC and PTC can occur as concurrent and synchronous tumours, which are anatomically distinct. When MTC and PTC merge, they form a so-called collision tumour. In mixed medullary-follicular (papillary) carcinoma (MMFC), both components are intimately intermixed and display morphological and immunohistochemical features of both origins within the same lesion. MMFCs are extremely rare, representing 0.15% of all thyroid tumours [102]. LNM tend to display a mixture of both histological components within the same lymph node [103]. In mixed MMFC, both components are intimately intermixed and display morphological and immunohistochemical features of both origins within the same lesion.

One group of patients with concurrent MTC and PTC showed a high incidence of presumed hereditary MTC (familial MTC) [104]. In other such cases, the co-expression of calcitonin and thyroglobulin was observed in the tumour cells at both protein and mRNA level, suggesting the possibility of their common origin from stem cell differentiation [103]. However, this hypothesis, along with the confirmation of the embryologic pathway, has not yet been validated.

Volante et al. [105] examined 12 MMFC cases for mutations in the *RET* proto-oncogene, allelic losses of 9 loci on 6 chromosomes, and clonal composition (X-chromosomal inactivation). Their results provided strong evidence that the follicular and medullary components in MMFCs were not derived from a single progenitor cell. Moreover, these tumours consistently presented different mutation patterns, allelic losses, and clonal composition in the 2 histological components. Based on these results, the authors proposed the “hostage hypothesis,” by which the neoplastic transformation of C cells leads to the development of MTC with entrapped non-neoplastic follicles, which are then transformed into hyperplastic follicular foci under the stimulation of trophic factors. Subsequent acquired genetic defects in follicular cells would then result in neoplastic transformation and the development of papillary or follicular neoplasms [102].

MTC may occur sporadically or as a component of inherited multiple endocrine neoplasia syndrome type 2 (20–30% of cases). Germline gain-of-function mutations in the *RET* proto-oncogene are associated with MTC de-

velopment, and somatic *RET* mutations are detected in about 50% of sporadic MTC cases [106]. Recent studies have shown that *RAS* somatic mutations were highly present in *RET*-negative tumours, suggesting *RAS* mutations as a possible alternative genetic event in sporadic MTC tumorigenesis [106, 107]. In general, *RET* and *RAS* mutations are mutually exclusive; however, both mutations have been detected in some cases of sporadic MTC [106]. There is no evidence of the presence of *RET* and/or *RAS* mutations in different subclones of MTC cancer cells or if they are related to the accumulation of oncogene mutations during cancer growth. Heterogeneity among subpopulations of sporadic MTC was observed in an early molecular study conducted in 1996 by Eng et al. [108]. Their analysis of primary tumours and metastases revealed that approximately 80% of sporadic MTC cases demonstrated at least 1 tumour cell subpopulation with a mutation in codon 918 of the *RET* proto-oncogene. The authors also suggested that the frequency of somatic mutations at *RET* codon 918 depended on the region of the tumour from which the material for molecular analysis was obtained. Mutations in the *RET* gene may arise during clonal evolution or, in the light of recent cancer stem cell theory, they could be related to the presence of other clones presenting this mutation.

Conclusion

The heterogeneity of TCs represents a clinical challenge, especially considering the intratumour variability. The studies conducted to date demonstrate a wide spectrum of mutations, representing “drivers” and “passengers” that may be used as molecular targets in targeted therapies. Although some data point to a clonal origin of the main TC mutations, there is contradictory evidence pointing to the subclonality of these alterations and the coexistence of different mutations within a single tumour. The development of drugs that would be effective for each separate tumour clone is a rather impossible feat because of treatment-related toxicity. Thus, the most optimal approach at present appears to be one that focuses on the primary driving mutation that is present in the whole tumour cell population. Another solution involves inhibition of the key signalling pathways participating in neoplastic transformation and cancer progression. The high intratumour heterogeneity of TC is also related to the development of drug resistance. Moreover, most of the currently available chemotherapeutic drugs aim at killing actively dividing tumour cells. Although 80% of

the cells on the tumour surface are dividing, Bhatia et al. [109] demonstrated that >90% of cells within a tumour are in a resting state.

Given the complexity of inter- and intratumour heterogeneity, pathologists should become familiar with the various heterogeneity levels to establish a clinically relevant diagnosis and collect high-quality material for further molecular studies. Gaining a deeper understanding of tumour heterogeneity will provide new possibilities to properly stratify patient risk, select markers for more ef-

fective therapeutic approaches, and bring individualised treatment to the forefront as the standard management approach for cancer patients.

Acknowledgements

This work was supported by the National Centre for Research and Development project under the program "Prevention practices and treatment of civilization diseases" STRATEGMED (STRATEGMED2/267398 /4/NCBR/2015).

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