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Application of molecular biology of differentiated thyroid cancer for clinical prognostication

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Abstract

Although cancer outcome results from the interplay between genetics and environment, researchers are making a great effort for applying molecular biology in the prognostication of differentiated thyroid cancer (DTC). Nevertheless, role of molecular characterisation in the prognostic setting of DTC is still nebulous. Among the most common and well-characterised genetic alterations related to DTC, including mutations of BRAF and RAS and RET rearrangements, $BRAF^{V600E}$ is the only mutation showing unequivocal association with clinical outcome. Unfortunately, its accuracy is strongly limited by low specificity. Recently, the introduction of next-generation sequencing techniques led to the identification of TERT promoter and TP53 mutations in DTC. These genetic abnormalities may identify a small subgroup of tumours with highly aggressive behaviour, thus improving specificity of molecular prognostication. Although knowledge of prognostic significance of TP53 mutations is still anecdotal, mutations of the TERT promoter have showed clear association with clinical outcome. Nevertheless, this genetic marker needs to be analysed according to a multigenetic model, as its prognostic effect becomes negligible when present in isolation. Given that any genetic alteration has demonstrated, taken alone, enough specificity, the co-occurrence of driving mutations is emerging as an independent genetic signature of aggressiveness, with possible future application in clinical practice. DTC prognostication may be empowered in the near future by non-tissue molecular prognosticators, including circulating $BRAF^{V600E}$ and miRNAs. Although promising, use of these markers needs to be refined by the technical sight, and the actual prognostic value is still yet to be validated.

Key Words
- thyroid carcinoma
- thyroid nodules
- molecular genetics
- environment

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Introduction

Prediction of clinical outcome in differentiated thyroid cancer

Thyroid cancer represents the most common endocrine malignancy showing an incidence of 14.3 per 10,000 inhabitants in the United States (Davies & Welch 2014). Differentiated thyroid cancer (DTC), including papillary (PTC) and follicular (FTC) histotypes, arises from epithelial follicular cells (Schlumberger 1998) and accounts for the vast majority (90%) of thyroid malignancies (Sherman 2003). During the last decades, incidence of DTC has

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progressively increased worldwide, including Western countries (Albores-Saavedra *et al*. 2007, Dal Maso *et al*. 2011, Davies & Welch 2014) and Asian population. Despite the raising morbidity, mortality due to thyroid cancer is stationary (Davies & Welch 2014, Oh *et al*. 2015). Indeed, prognosis of patients affected with DTC is typically favourable with a 10-year disease-related survival of 85% (Eustatia-Rutten *et al*. 2006). This is due to both the intrinsic indolent behaviour of the disease (Schlumberger 1998) and the efficacy of initial treatment, consisting in total/near-total thyroidectomy and, in selected cases, radioactive iodine (RAI), followed by the suppression of thyroid-stimulating hormone (TSH) (Haugen *et al*. 2016). The low mortality related to DTC makes it difficult to perform prognostic studies with overall survival as the primary endpoint because long-term follow-up is needed to achieve it. By contrast, the persistence of structural disease after initial treatment or the development of recurrences after complete remission has been reported in about 25–30% of the patients (Tuttle *et al*. 2010*b*, Vaisman *et al*. 2012, Castagna *et al*. 2011, Pitoia *et al*. 2013) (Fig. 1). Importantly, these parameters are strictly related to disease-specific survival (Mazzaferri & Jhiang 1994, Tuttle *et al*. 2010*a*, Brown *et al*. 2011) and can therefore be represented as valid prognostic endpoints. Hence, the rate of persistent/recurrent disease and the disease-free status, if survival analyses were performed, represent more feasible parameters to be considered when assessing prognosis in DTC and are used as the primary endpoints in most prognostic studies about this clinical setting. Given that the AJCC/UICC system was able to predict mortality but not persistence/recurrence (Orlov *et al*. 2009, Baek *et al*. 2010, Tuttle *et al*. 2010*b*, Vaisman *et al*. 2012), a great effort has been made in the last decade to build novel staging systems specifically dedicated to the prediction of persistent/recurrent disease. Particularly, each of the major societies dealing with thyroid diseases (ATA (American Thyroid Association), ETA (European Thyroid Association) and LATS (Latin American Thyroid Society)) has validated a categorical classification identifying subgroups with different risks of persistent/recurrent disease (Pacini *et al*. 2006, Pitoia *et al*. 2009, 2013). Nevertheless, the long-term risk stratification obtained by the mentioned systems is still suboptimal. First, all of them showed a proportion of variance explained, a statistical measure which analyses the capability of a staging system to predict the outcome of interest (Schemper & Stare 1996), of less than 30% (Momesso & Tuttle 2014). Even more importantly, they demonstrated a low positive predictive value (PPV) (Castagna *et al*. 2011). This represents a crucial limit as the identification of that subgroup of persisting/recurring DTC subjects represents the main goal of prognostic stratification, with possible dramatic impact on clinical management. To refine the risk estimate of persistent/recurrent disease, recent guidelines from the ATA have introduced a personalised non-categorical model based on the concept of 'continuum of risk', where a wider range of variables were used to perfectly fit individual features of each patient and provide a quantitative determination of the risk (Haugen *et al*. 2016). Nevertheless, only the identification and validation of novel prognosticators with high specificity and therefore PPV of disease persistence/recurrence may allow overcoming the current limits of DTC prognostic system.

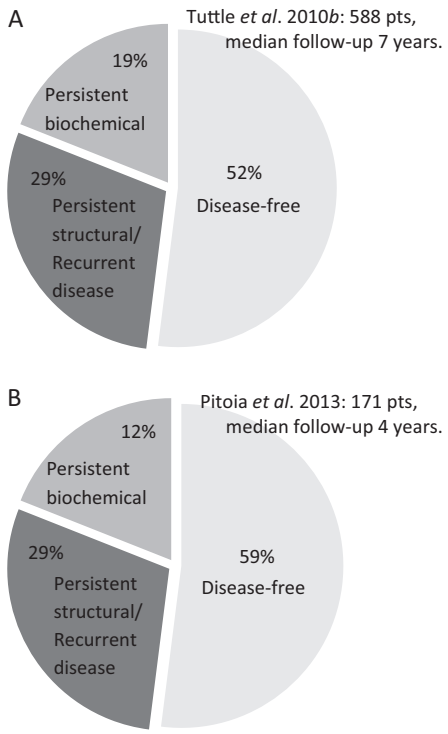

Figure 1
Clinical outcome of differentiated thyroid cancer in 2 large cohorts. Data from Tuttle *et al*. (2010*b*), and Pitoia *et al*. (2013) (A and B, respectively).

Molecular genetics of thyroid cancer and current clinical applications

A wide body of research has been performed within the last decades to improve the knowledge of molecular pathogenesis of DTC. This led to the identification of a set of molecular alterations with demonstrated/putative pathogenetic role (Xing 2013). These abnormalities are heterogeneous, including both genetic (gene mutations,

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translocations, amplifications and copy number gains) and epigenetic (aberrant gene methylation) alterations, which involve the MAP kinases and the phosphatidylinositol-3 kinase (PI3K)/Akt/mTOR signalling cascades (Kondo *et al*. 2006, Nikiforov & Nikiforova 2011). Based on the identified set of molecular abnormalities, the Thyroid Cancer Genome Atlas (TCGA) has recently performed a comprehensive genetic and epigenetic analysis of a large cohort of PTC samples by means of the most innovative next-generation sequencing (NGS) techniques (Agrawal *et al*. 2014). Importantly, a great effort was made for attesting the driver role of identified mutations. This represented the most accurate attempt of molecular characterisation of PTC performed to date, leading to the identification of genetic abnormalities with founder significance in the vast majority of analysed tumours. Particularly, the absence of any putative pathogenic molecular alteration was reported in less than 4% of cases (the so-called dark matter) (Fig. 2), meaning that current knowledge of molecular genetics of DTC is so advanced as to almost cover the totality of patients. The increasing knowledge on molecular features related to DTC has been constantly accompanied by the effort to convert biological discoveries into clinical applications. To date, molecular characterisation of DTC has found its main clinical application in the diagnostic setting. Since the beginning of 2000s, several authors tried to use genetic findings for improving pre-surgical diagnosis of thyroid nodules showing indeterminate cytology (Cheung *et al*. 2001, Xing *et al*. 2004, Sapio *et al*. 2007, Nikiforov *et al*. 2009). During subsequent years, the continuous discovery of genetic abnormalities related to DTC and the development of the NGS techniques, which allowed the simultaneous analysis of a large number of molecular alterations, led to the development of mutational panels specifically dedicated to pre-surgical diagnosis of DTC on cytology samples. This body of research culminated

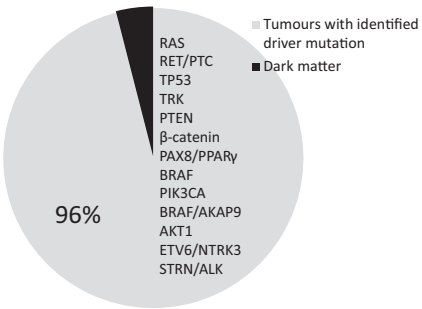

Figure 2
Current status of molecular characterisation of differentiated thyroid cancer.

in the recent validation of an expanded NGS panel, termed ThyroSeq, including 15 genes, which demonstrated high accuracy in terms of both sensitivity and specificity for the diagnosis of cancer in patients with thyroid nodules showing indeterminate cytology (Nikiforova *et al*. 2013, Nikiforov *et al*. 2014). To date, molecular analysis of cytology specimens is slowly but progressively entering into clinical practice. Although further validation studies are needed to place molecular testing in a defined work-up algorithm, the latest ATA guidelines (Haugen *et al*. 2016) indicated this approach as a feasible option for a supplemental determination of the malignancy risk in case of indeterminate cytology. By contrast, application of molecular characterisation in the prognostic setting of DTC is still at a preliminary level. To date, any molecular marker has a well-defined role in the risk stratification of DTC, and the introduction of the so-called molecular prognostication into 'real-life' clinical practice is still yet to be performed. In this review, we will analyse the current knowledge about prognostic significance and the actual role of the most common and best studied genetic alterations related to DTC, including BRAF and RAS point mutations and RET/PTC rearrangements, in the prognostic setting (Nikiforov & Nikiforova 2011, Xing 2013). Afterwards, we will discuss about the recent findings and possible prognostic role of some emerging molecular markers, specifically focusing on TERT promoter and TP53 mutations and on the co-occurrence of driver mutations, which is considered as an independent genetic feature. Finally, we will discuss about the current evidence and possible future application in the prognostic setting of non-tissue molecular markers.

RET rearrangements

RET rearrangements include a group of chimeric oncogenes, with RET/PTC-1 and -3 variants being the most frequent, generated by the fusion of the catalytic domain of the tyrosine kinase receptor *RET* to the 5′ terminal region of heterologous genes (Santoro *et al*. 2006). RET/PTC exclusively occurs in the thyroid gland (Nikiforova *et al*. 2000). Regarding thyroid malignancies, the mutation is specifically associated with PTC, with higher occurrence in the classic variant than that in the follicular variant (Lam *et al*. 1998). Pathogenetic role of RET rearrangements in PTC has been elaborately described (Santoro *et al*. 1993, Jhiang *et al*. 1998, Tallini *et al*. 1998, Powell *et al*. 1998). Nevertheless, the TCGA study reported the occurrence of RET/PTC as a founder genetic event in only 6.8% of the PTC cohort (Agrawal *et al*. 2014). This represented a breakthrough, as estimation of actual prevalence (Zhu

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et al. 2006, Marotta *et al*. 2011*a*) and biological significance (Guerra *et al*. 2011) of the mutation had been hampered by the application of different detection methods. The low percentage of tumours having RET rearrangements as driver mutational event represents a crucial limitation for clinical application of the mutation, both in the diagnostic and the prognostic setting. However, prognostic significance of RET/PTC is still controversial. The major point to be considered when assessing the characteristics of PTC harbouring RET rearrangements is the presence of two separate entities, namely spontaneous and radiation-induced tumours, broadly differing in both clinical and genetic features. Indeed, studies in Belarus, Ukraine and parts of the Russian Federation evidenced that Chernobyl radiation exposure induced a dramatic increase in the number of thyroid cancers of PTC type in childhood and that tumours harboured RET rearrangements with higher frequency than with spontaneous PTCs (Nikiforov 2002). Importantly, post-Chernobyl PTC showed more aggressive clinico-pathological features, including diffuse intra-thyroidal dissemination, invasion of the capsule and adjacent soft tissue, lymph node extension, and a high prevalence of the aggressive solid variant (Nikiforov & Gnepp 1994). Comparative analyses of specific RET/PTC variants revealed that RET/PTC-1 was dominant within spontaneous forms where it was strongly related to the classic variant, whereas RET/PTC-3 was predominant among radiation-induced carcinomas where an association with the solid variant was demonstrated (Nikiforov *et al*. 1997, Tallini *et al*. 1998, Thomas *et al*. 1999, Rabes *et al*. 2000). Furthermore, RET/PTC-3 was more frequent than RET/PTC-1 in tall cell PTC, which represents the most aggressive variant (Ghossein *et al*. 2007, Milione & Seregni 2010), and its expression in transgenic mice generated solid PTC with metastatic spread (Santoro *et al*. 1996, Powell *et al*. 1998). Therefore, a different oncogenic potential, likely determining an opposite prognostic impact, has been proposed for RET/PTC-1 and -3. This hypothesis was further empowered by Rabes and coworkers (2000) who reported that tumours with RET/PTC-3 had a shorter latency and a more aggressive clinical behaviour, compared with those carrying RET/PTC-1. However, PTC related to radiation exposure can be considered as a didactic model of environment-determined tumour oncogenesis, but is not representative of 'real life'. Therefore, application of RET/PTC as a prognostic marker in clinical practice should only derive from the discovery of an association with clinical outcome in the setting of spontaneous tumours. Studies specifically focusing on spontaneous PTC suggested that RET/PTC-1, the most frequent RET rearrangement found in this setting, was associated with more favourable behaviour (Nikiforov 2004) and that tumours harbouring the mutation had a very low probability of progression to poorly differentiated and undifferentiated carcinomas, compared with those carrying BRAF and RAS mutations (Mayr *et al*. 1997, Soares *et al*. 1998, Tallini *et al*. 1998). Nevertheless, pre-clinical studies identified RET/PTC as a weak tumour-initiating factor and suggested that secondary genetic or epigenetic changes were required for full neoplastic transformation (Powell *et al*. 1998, Wang *et al*. 2003). To date, the most accepted thesis is that RET/PTC has low oncogenic potential in spontaneous PTC and may play a role in tumour initiation, but not progression. This is also supported by the finding of a higher occurrence of the mutation in micro-PTC, compared with clinically evident tumours (Sugg *et al*. 1998, Tallini *et al*. 1998, Fusco *et al*. 2002). These observations make a relevant prognostic impact on RET rearrangements in spontaneous PTC unlikely. Another important issue to be taken into consideration is that detection of RET rearrangements is strongly affected by the sensitivity of the detection method. Indeed, the introduction of more sensitive techniques, such as southern blot on RT/PCR products and FISH, led to the detection of higher prevalence of RET rearrangements in PTC (Elisei *et al*. 2001, Guerra *et al*. 2011) as compared with older studies using less accurate approaches (Santoro *et al*. 1992, Jhiang *et al*. 1998). The crucial role of the method was clearly demonstrated by Zhu and coworkers (2006) who applied different techniques for detecting RET rearrangements in the same cohort of PTC, demonstrating broad variability as a result of the different analytic sensitivity. The use of highly sensitive techniques also allowed the detection of non-clonal mutational events, namely the presence of RET rearrangements in a small proportion of tumour cells or even in a single cell. This paradoxically hampered the possible clinical application of the mutation as molecular marker, in both diagnostic and prognostic settings (Marotta *et al*. 2011*a*). Indeed, several authors reported the presence of RET/PTC in benign thyroid diseases, including not only Hashimoto's thyroiditis (Wirtschafter *et al*. 1997, Sheils *et al*. 2000, Rhoden *et al*. 2006) but also thyroid nodules revealing benign histology (Cinti *et al*. 2000, Elisei *et al*. 2001, Guerra *et al*. 2011). This posed the question of a possible different biological significance of non-clonal occurrence of RET/PTC, compared with clonal mutation (Marotta *et al*. 2010*a*). Some recent papers demonstrated

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that benign nodules with non-clonal RET/PTC occurrence had more rapid volume increase, compared with lesions not harbouring the mutation (Marotta *et al*. 2010*b*, Sapio *et al*. 2011). This may suggest specific biological and therefore clinical significance for non-clonal RET/PTC in malignant disease also. To conclude, given the apparently weak association of the mutation with clinical outcome and the possible biological difference between clonal and non-clonal mutation, which needs to be further defined, RET/PTC has no current role in the prognostic stratification of PTC.

BRAF^{V600E}

The T1799A transverse point mutation of the proto-oncogene BRAF, resulting in the valine-to-glutamate (V600E) amino acid substitution, is nearly the only BRAF mutation found in thyroid cancer, with a very few exceptions of the K601E and A598V missense mutations, the AKAP9/BRAF recombination, the 1799–1801 deletion and the 1799–1816 insertion (Ciampi *et al*. 2005, Xing *et al*. 2005, Hou *et al*. 2007*b*, Santarpia *et al*. 2009). Pathogenetic role of the mutation has been widely proved by pre-clinical studies (Knauf *et al*. 2005, Liu *et al*. 2007). According to the TCGA, BRAF^{V600E} largely represents the most common driver mutational event involved in PTC (58.5% of the cohort) (Agrawal *et al*. 2014). Unlike RET rearrangements, several authors reported a clear association of BRAF^{V600E} with molecular features suggestive of biological and clinical aggressiveness. Particularly, the mutation was associated with decreased or absent expression of thyroid iodide-handling genes (the sodium-iodide symporter, the TSH receptor, the pendrin gene (SLC26A4), the thyroperoxidase and the thyroglobulin) (Durante *et al*. 2007, Xing 2007), whose expression was demonstrated to be strictly dependent on that of BRAF^{V600E} (Liu *et al*. 2010, Chakravarty *et al*. 2011). Furthermore, *BRAF* mutation was associated with the overexpression of various tumour-promoting factors, such as VEGF and MET (Xing 2007). From the clinico-pathological perspective, BRAF^{V600E} was associated with the aggressive tall cell variant of PTC. Although still controversial, the majority of studies also reported the association of mutated BRAF with several other clinico-pathological features having negative prognostic impact, such as lymph node metastases, extrathyroidal extension and advanced disease stage (Xing *et al*. 2005, Kebebew *et al*. 2007, Lee *et al*. 2007, Frasca *et al*. 2008, Wang *et al*. 2008). Owing to this body of evidence, BRAF^{V600E} has been considered the best candidate as molecular prognosticator of PTC, and several prognostic studies have been dedicated to assess its relationship with clinical outcome. After a wide series of single-centre studies showing controversial results, 2 large multicentre cohorts have been recently analysed for assessing the impact of BRAF mutation on mortality and recurrence, respectively (Fig. 3). The first paper including 1849 patients showed the association of mutated BRAF with increased disease-specific mortality on univariate analysis (Xing *et al*. 2013). More importantly, the second one including 2099 patients demonstrated an independent association between *BRAF* mutation and recurrent disease both in the overall PTC population and after stratification for histotypes (classic and follicular variant) (Xing *et al*. 2015). Despite the unequivocal

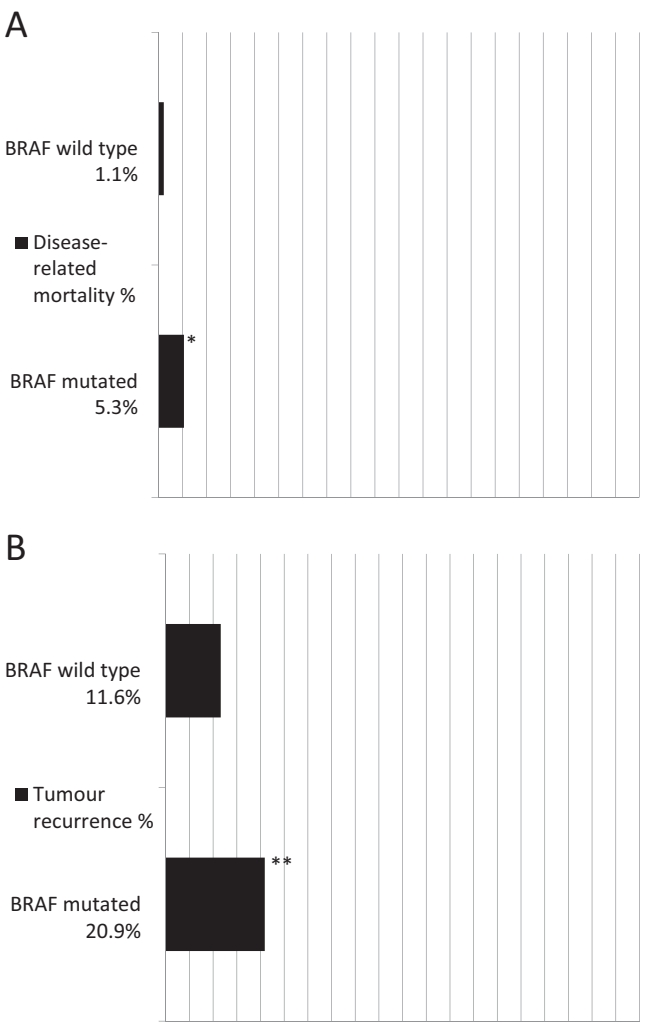

Figure 3
Association between BRAF^{V600E} and tumour-related mortality (A) and recurrence (B) in 2 large multicentre cohorts. Data from Xing *et al*. (2013) and Xing *et al*. (2015), respectively. *Significantly different at univariate analysis; **Significantly different at multivariate analysis.

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association with disease recurrence, clinical application of BRAF^{V600E} as prognostic marker is hampered by its low specificity. Indeed, analysis from the largest meta-analysis available to date (2167 patients) showed acceptable sensitivity (65%), but poor specificity for the prediction of recurrent disease with a PPV of only 25% (Tufano *et al.* 2012). Thus, current role of mutated BRAF for the risk stratification of PTC is limited, as it is unlikely to be used in isolation, but only in a multivariable context, combined with other prognostic features. To date, the 2015 ATA guidelines do not suggest the routine determination of BRAF status, but consider BRAF^{V600E} as an information to be included (if present) for the risk estimate of recurrent disease in ATA low-risk patients according to the 'continuum of risk' model (Haugen *et al.* 2016). In recent years, a wide body of research has been performed to attest whether BRAF^{V600E} was a clonal or sub-clonal mutational event. This represents a crucial issue with possible dramatic impact not only on the biological perspective but also on clinical implications related to the mutation, with the inclusion of the prognostic role. Particularly, 2 studies (Guerra *et al.* 2012*b*, Gandolfi *et al.* 2013) opened a burning issue among researchers dealing with thyroid carcinogenesis. In both papers, authors searched for BRAF^{V600E} in PTC samples by means of pyrosequencing, a sequencing-by-synthesis method providing the exact percentage of alleles (and therefore cells) bearing the mutation (Ronaghi *et al.* 1998). Unexpectedly, both research groups found that mutated BRAF occurred sub-clonally in the majority of cases. Subsequently, Guerra and coworkers (2012*a*) also demonstrated that the percentage of mutated alleles significantly impacted on the risk of recurrence, further supporting the biologic significance of BRAF^{V600E} clonality. The heterogeneity of BRAF mutation in PTC is still a hot point of current research on thyroid cancer. In 2013, Fagin and his research group assessed BRAF^{V600E} expression by means of immunostaining for the mutation-specific antibody VE1 (Ghossein *et al.* 2013). Authors found that almost the totality (13 of 14 cases) of PTC with strong immunopositivity (all carrying the BRAF mutation) had homogeneous distribution of the staining, thus concluding that BRAF^{V600E} occurrence is a clonal event. More recently, de Biase and coworkers (de Biase *et al.* 2014*a*) assessed the percentage of BRAF-mutated alleles in a PTC series by means of modern and more accurate techniques, such as the allele-specific locked nucleic acid PCR and 454 NGS (Morandi *et al.* 2012, de Biase *et al.* 2014*b*). They confirmed the heterogeneity of the mutation, demonstrating that BRAF^{V600E} was a clonal event in less than 50% of cases. By contrast, the TCGA (Agrawal *et al.* 2014) used a dedicated software (ABSOLUTE package (Carter *et al.* 2012)) to calculate cancer cell fraction of the previously identified driver mutations with the inclusion of BRAF^{V600E}, finding that the majority of tumour cells harboured the mutations. Thus, the authors concluded that founder mutations are always clonal. Despite this result, it is our opinion that the issue of BRAF^{V600E} clonality in PTC is still open. A better understanding of this specific aspect related to BRAF mutation may improve the utility of the mutation in the prognostic setting. Indeed, the quantitative determination of BRAF^{V600E} may lead to the identification of a threshold of mutated alleles, above which patients show poorer prognosis. This could allow us to improve the specificity and therefore PPV of disease recurrence, thus overcoming the main limitation of qualitative BRAF^{V600E} determination.

RAS mutations

Point mutations of the RAS genes, including the 3 isoforms HRAS, KRAS and NRAS, historically represent the second most common genetic alterations of DTC (Xing 2013). Oncogenic power of these mutations in thyroid has been already demonstrated by *in vivo* and *in vitro* experimental studies (Bond *et al.* 1994, Rochefort *et al.* 1996). The TCGA study has identified RAS mutations as the driver molecular alteration of 12.7% of the PTC cohort. As reported previously (Suarez *et al.* 1990, Vasko *et al.* 2003), all possible mutants were detected, but alterations involving codon 61 of NRAS were most frequently found (3.5% HRAS, 0.7% KRAS and 8.5% NRAS) (Agrawal *et al.* 2014). RAS mutations are strictly related to follicular architecture of DTC. First, they are detected in a wide portion (40–50%) of FTC (Lemoine *et al.* 1989, Esapa *et al.* 1999). Furthermore, among the 10–20% of PTCs harbouring the mutations, almost all cases are classified as follicular variant (Zhu *et al.* 2003, Adeniran *et al.* 2006). Importantly, RAS alterations are not specific for thyroid malignant disease, occurring in 20–25% of follicular adenomas (Namba *et al.* 1990, Liu *et al.* 2008). Although follicular adenomas bearing mutated RAS are considered as lesions with high malignant potential (Gupta *et al.* 2013), this strongly hampers the use of RAS status as molecular marker, both in the diagnostic and prognostic setting. To date, prognostic significance of RAS mutations in DTC has been addressed in few studies, with controversial results. Nevertheless, available data slightly support a negative prognostic effect related to mutated RAS, with increased risk of tumour dedifferentiation and higher rates of distant metastases, recurrence and death

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(Karga *et al*. 1991, Hara *et al*. 1994, Manenti *et al*. 1994, Basolo *et al*. 2000, Garcia-Rostan *et al*. 2003, Volante *et al*. 2009, Fukahori *et al*. 2012). The most feasible theory is that aberrant RAS activation induced by the mutations may stimulate evolution from well-differentiated cancers to less differentiated forms, namely poorly differentiated and undifferentiated carcinomas, thus worsening prognosis. This hypothesis is supported by the finding that both PTC and FTC with poorly differentiated areas showed higher rates of mutated RAS (Nikiforova *et al*. 2003, Zhu *et al*. 2003). Furthermore, it has been demonstrated that mutant RAS determines chromosome instability (Saavedra *et al*. 2000), and this is consistent with its possible dedifferentiating effect. Nevertheless, all prognostic studies performed to date about mutant RAS in DTC rely on small sample size and include heterogeneous populations (DTC and less differentiated forms of thyroid cancer). Therefore, larger studies specifically focused on DTC are required for better understanding the prognostic value of RAS mutations and for verifying if determination of RAS status can have a role in prognostic stratification.

Emerging molecular prognosticators: TERT promoter and TP53 mutations

In recent years, novel molecular markers are breaking into molecular biology of thyroid cancer, and their possible clinical application is currently under evaluation. Particularly, mutations involving the promoter of the telomerase catalytic subunit telomerase reverse transcriptase (TERT) and the tumour suppressor TP53 are emerging as feasible tools for molecular prognostication of DTC. In the recent years, mutations of the TERT promoter, with the 1,295,228 C>T (C228T) and the 1,295,250 C>T (C250T) being the most commonly detected, have represented a hot topic of translational cancer research (Huang *et al*. 2013). Both mutations induce the formation of a consensus binding site for the ETS (E-twenty-six) transcription factors, thus leading to increased gene expression (Horn *et al*. 2013). This induces telomerase activation, inhibition of the physiological telomere shortening and immortalisation of cancer cells (Hanahan & Weinberg 2011). Importantly, telomerase activity was associated to advanced stage and extrathyroidal extension in DTC, and this is consistent with a possible role for TERT promoter mutations in tumour progression (Bornstein-Quevedo *et al*. 2003). Starting from the study by Liu and coworkers (2013), who firstly reported the mutations in follicular cell-derived thyroid cancers, a relevant body of research has been performed by 3 leading groups and by the TCGA, focusing on prevalence and possible prognostic implications of TERT promoter mutations in thyroid cancer (Agrawal *et al*. 2014, Melo *et al*. 2014, Liu *et al*. 2014*a*, Xing *et al*. 2014*b*). Recently, a comprehensive meta-analysis summarising all available data has been published (Liu & Xing 2016). Although mutations were more frequent in less differentiated tumours, a relevant portion of DTC was involved (11.3% (4.5–25.5) of PTC and 17.1% (13.8–36.4) of FTC). More importantly, TERT promoter mutations revealed association with both clinico-pathological features and outcome. In DTC, which represents the focus of our review, patients carrying the mutations were older, with larger tumours and higher rates of extrathyroidal extension and vascular invasion. Nevertheless, the strongest and more relevant association was found with distant metastases and advanced stage (III/IV), which represent the most relevant factors impacting on prognosis (Sampson *et al*. 2007). Besides the relationship with clinico-pathological features, which provides partial prognostic information, genetic alterations of TERT promoter directly predicted clinical outcome in a large number of patients, where a strong association was demonstrated with both recurrence and mortality. This body of evidence led a wide part of literature dealing with DTC to categorise prognostic effect of TERT promoter mutations as dramatic, even overcoming prognostic performance of the $BRAF^{V600E}$ oncogene, which was historically considered as the best molecular prognosticator (as discussed previously). Nevertheless, this was misleading as recently demonstrated by a series of studies performing simultaneous analysis of TERT promoter and BRAF (Xing *et al*. 2014*a*,*b*, Liu *et al*. 2014*b*, Song *et al*. 2016). Indeed, all these papers consistently showed that prognostic effect related to alterations of TERT promoter disappeared or strikingly decreased when mutations occurred separately (Fig. 4), suggesting that actual prognostic value of the genetic marker had been overestimated and co-existence of BRAF mutation was mandatory for promoting tumour aggressiveness. Recently, Vinagre and coworkers (2013) found higher TERT mRNA expression in those PTCs harbouring both TERT promoter mutations and $BRAF^{V600E}$, compared with those carrying one of the genetic alterations separately. Therefore, it is conceivable that $BRAF^{V600E}$ may upregulate the ETS system through the activation of the MAP kinases cascade (Whitmarsh *et al*. 1995), thus leading to TERT overexpression. This may further enhance telomerase activity, thus amplifying the oncogenic power related to TERT promoter mutations. Therefore, actual biological role and prognostic significance of genetic alterations

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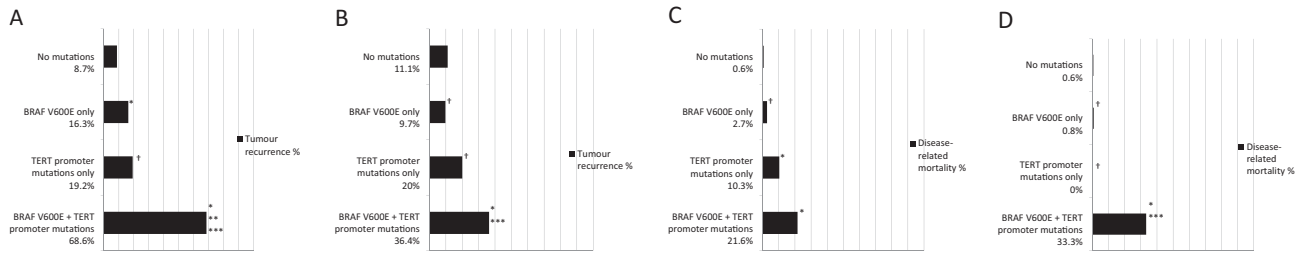

Figure 4
Synergistic prognostic effect of co-occurrence of BRAF V600E and TERT promoter mutations in increasing the risk of tumour recurrence (A and B) and disease-related mortality (C and D) in DTC. Data from Xing *et al*. (2014*b*) (A), Xing *et al*. (2014*a*) (C), and Song *et al*. (2016) (B and D). †Not significantly different from the *No mutations* group; *Significantly different from the *No mutations* group; **Significantly different from the *TERT promoter mutations only* group; *** Significantly different from the *BRAF V600E only* group.

of TERT promoter needs to be considered as inserted in a wider mutational context, taking into consideration the co-occurrence of other molecular abnormalities, including $BRAF^{V600E}$ and likely RAS mutations. However, a more in-depth analysis of prognostic implications related to the simultaneous occurrence of genetic alterations involving TERT promoter, BRAF and RAS is given in the 'Co-occurrence of driver mutations' section below. Although typically considered as a marker of tumour dedifferentiation and detected in a wide portion of poorly differentiated or undifferentiated thyroid cancer (Donghi *et al*. 1993, Fagin *et al*. 1993), recent mutational analysis by means of NGS has also identified TP53 mutations in a low percentage of DTC, namely 3.5% of PTC and 11% of oncocytic FTC (Nikiforova *et al*. 2013). Even more importantly, authors reported a more aggressive clinical behaviour for this little subgroup of TP53-mutated tumours, thus suggesting the possible application of the mutation in the prognostic setting. More recently, a study by TCGA has confirmed the involvement of TP53 in PTC, but the prevalence of the mutation was even lower than what was reported in the study by Nikiforova, with only 3 positive patients (0.7%) (Agrawal *et al*. 2014). Unfortunately, no prognostic information can be extrapolated from the TCGA as data about clinico-pathological features and outcome of patients carrying the mutation were largely insufficient. Besides the study by Nikiforova, which was based on a small sample size, data about relationship of TP53 mutations and/or p53 (which represents the gene product) expression with characteristics of DTC remain mainly anecdotal, with some reports associating p53 overexpression to aggressive PTC variants such as the columnar, tall cell and cribriform–morular (Putti & Bhuiya 2000, Cameselle-Teijeiro *et al*. 2009). Therefore, dedicated studies are required to assess the clinico-pathological features and outcome of TP53-mutated DTC, thus refining the actual prognostic value of the mutation. Ultimately, both TERT promoter and TP53 mutations may be useful for the identification of a small subgroup of highly aggressive tumours, thus improving the specificity of molecular prognostication of DTC.

Co-occurrence of driver mutations

Up to now, no genetic alteration has demonstrated, taken alone, enough specificity for the identification of persistent/recurrent disease in DTC. Until recently, co-occurrence of genetic abnormalities had been exclusively reported in undifferentiated thyroid cancer (Garcia-Rostan *et al*. 2005, Hou *et al*. 2007*a*). This led the majority of authors dealing with thyroid cancer to consider the mutual exclusivity of genetic alterations as a paradigm of DTC oncogenesis. In the recent years, this concept was toppled. In 2008, Liu and coworkers (2008) found co-existence of genetic alterations, involving gene mutations and copy number gains, not only in undifferentiated thyroid cancer but also in FTC. Therefore, authors suggested that occurrence of multiple genetic hits is required for DTC development. Nevertheless, in the study by Liu, combination of genetic abnormalities detected in the FTC mainly involved copy number gains, whereas gene mutations were largely mutually exclusive. More recently, the NGS analysis by Nikiforova and coworkers (2013) reported the co-occurrence of gene mutations in a small but significant portion, namely 4%, of DTC. Even more importantly, authors found that this mutational status was associated with aggressive behaviour, particularly the presence of distant metastases. Following these findings, recent research dealing with DTC oncogenesis has focused on the prognostic significance of a particular combined mutational status, namely the association between the $BRAF^{V600E}$ oncogene and TERT promoter mutations.

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Particularly, strong evidence has been achieved about the deleterious prognostic effect related to this combination. Xing and his team were the first researchers to demonstrate that simultaneous occurrence of $BRAF^{V600E}$ and the TERT promoter mutation C228T, which is the most largely detected in DTC (Liu & Xing 2016), is more strongly associated to high-risk clinico-pathological features and to the development of tumour recurrence (Fig. 4A), compared with the presence of one of the mutations separately (Xing *et al*. 2014*b*). Subsequently, authors also demonstrated similar synergistic interplay in worsening disease-related mortality (Xing *et al*. 2014*a*) (Fig. 4C). More recently, consistent results have been obtained by Song and coworkers (2016) (Fig. 4B, C and D), who included both C228T and C250T mutations in the analysis. Based on these findings, the association between $BRAF^{V600E}$ and TERT promoter mutations has to be considered as a unique genetic hallmark, identifying a subgroup of DTC patients with aggressive disease and poor prognosis. To date, still limited but significant data exist about possible interplay between TERT promoter and RAS alterations in affecting DTC prognosis. In 2015, Muzza and coworkers (2015) were the first researchers to focus on this aspect. They found that the 2 molecular abnormalities were synergic in increasing the risk of persistent disease, but failed to demonstrate similar interaction for the risk of tumour recurrence. More recently, the aforementioned study by Song (Song *et al*. 2016) showed a synergistic effect between RAS and TERT promoter mutations in worsening clinico-pathological features and outcome of DTC patients, including both tumour recurrence and disease-related mortality. Although further studies are needed to assess the actual interplay with RAS status, the presence of TERT promoter mutations may allow the identification of a subgroup of aggressive tumours within both BRAF- and RAS-mutated DTC, which represent the main clinico-molecular types (Agrawal *et al*. 2014). At present, it is difficult to establish which of these mutations occurs earlier. It is conceivable that BRAF and RAS alterations may act as early mutational events, whereas *TERT* promoter mutations may represent a late genetic hit, increasing the proliferative potential of cancer cells and therefore stimulating disease progression. The hypothesis of the late occurrence of TERT promoter mutations as a key point for tumour progression in DTC is sustained by a specific finding from the study by Song (Song *et al*. 2016), as authors demonstrated that mutations significantly worsened the prognosis of patients with advanced disease. This effect was independent from BRAF and RAS status. Nevertheless, it cannot be excluded that TERT promoter alterations may also act as early genetic hit, as mutations have been detected across all stages and grades in most cancers (Chiba *et al*. 2015). Indeed, mutations are detected alone in a small but non-negligible portion of DTC, even if prognostic effect is lost or extremely mild in this case, as already discussed in the previous paragraph. However, pre-clinical studies addressing the molecular interplay between different genetic events are required to better understand the biological role of TERT promoter mutations in DTC oncogenesis. Owing to this body of evidence, the latest ATA guidelines (Haugen *et al*. 2016) considered the combination of mutations involving multiple founder genes as an independent genetic signature of aggressiveness, which allows the identification of a small subgroup of tumours with extremely aggressive behaviour.

Non-tissue prognosticators

To date, molecular analysis of DTC, and therefore molecular prognostication, is exclusively based on tissue markers. This is a limitation as tumour tissue, including not only surgical samples but also fine-needle ago-biopsy specimens, is not always available. Furthermore, a different mutational status may occur in metastatic sites compared with primary tumour. Therefore, molecular characterisation from primary tumour may provide outmoded and misleading information. Therefore, the identification of non-tissue markers may facilitate and empower molecular prognostication of DTC. Given that $BRAF^{V600E}$ is the more frequent somatic mutation and the main prognosticator of DTC, several authors searched for the mutation in circulating free DNA (Marotta *et al*. 2011*b*). First, Chuang and coworkers (2010) analysed the serum from a small series of patients, demonstrating that 60% of cases who were positive for $BRAF^{V600E}$ in primary tumours also had detectable circulating BRAF mutation. Afterwards, Cradic and coworkers (2009) investigated whether BRAF mutation could be detected in the blood of patients with residual or metastatic disease, finding the mutation in 21% of cases. By contrast, recent data failed to detect circulating $BRAF^{V600E}$ in 94 serum samples from patients with PTC harbouring the mutation at the somatic level using a quantitative PCR method (Kwak *et al*. 2013). This discrepancy could be related to the use of assay reagents with inadequate sensitivity and/or not optimised for plasma samples in addition to uncontrolled pre-analytical steps. Recently, research by Pupilli and coworkers (2013) further empowered the use of circulating BRAF mutation as biomarkers in DTC. Authors demonstrated that the

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percentage of $BRAF^{V600E}$ detected in the serum increased progressively across cytological categories, being higher in patients with histologically confirmed PTC compared with those with benign histology. Furthermore, analysis of the mutation before and after treatment clearly indicates an association between the mutation and the presence of active disease. Thus, BRAF mutation detected in circulating DNA represents a promising tool to be specifically analysed in the prognostic setting. Molecular prognostication of DTC may be further improved by the use of microRNAs (miRNAs), which are short (about 19–22 nucleotides), non-coding RNA sequences having relevant role in cancer development and progression through their regulatory activity on gene expression at both the transcriptional and post-transcriptional levels (Calin *et al.* 2002, Ma *et al.* 2007). To date, dysregulation of several miRNAs has been demonstrated in PTC, and the actual prognostic implication of these genetic features represents a hot point of current research (Chruscik & Lam 2015). Possible prognostic role of miRNA patterns in PTC was suggested in a breakthrough study by Gao and coworkers (2010). They reported different miRNAs expression between cell lines subpopulations from human PTC with lymph node involvement showing increasing metastatic potency, compared with their control subpopulations. As consistently reported by a wide set of studies (He *et al.* 2005, Pallante *et al.* 2006, Tetzlaff *et al.* 2007, Nikiforova *et al.* 2008, Swierniak *et al.* 2013), upregulation of miR-146b and the miR-221/miR-222 cluster represent the most frequent miRNA alterations related to PTC, and this has been definitely confirmed by the recent deep-sequencing analysis of the largest cohort assessed to date (Mancikova *et al.* 2015). Therefore, many authors have searched for a possible association between the mentioned miRNAs and clinical outcome in DTC. First, Chou and coworkers showed that BRAF-mutated PTC, having a recognised more aggressive behaviour, had higher miR-146b expression compared with those not carrying the oncogene (Chou *et al.* 2010). Afterwards, the same research group performed a follow-up study demonstrating poorer overall survival among patients with high levels of miR-146b (Chou *et al.* 2013). Two research groups found an association between miR-146b and miR-222 overexpression and distant metastasis, recurrence and BRAF expression (Yip *et al.* 2011, Lee *et al.* 2013). Furthermore, Zhou and coworkers found that overexpression of miR-221 was associated with extrathyroidal extension, lymph node metastasis, advanced disease stages and BRAF mutation (Zhou *et al.* 2012). An emerging miRNA with possible prognostic application in DTC is miR-205. Indeed, Salajegheh and coworkers recently demonstrated an underexpression of miR-205 in DTC specimens, compared with that in normal tissues, and, more importantly, associated the entity of the dysregulation to distant metastases and advanced stages (Salajegheh *et al.* 2015). In the same paper, authors also provided pre-clinical support to their finding demonstrating both anti-angiogenic and tumour-suppressive action of miR-205 in thyroid cancer cell lines. Thus, underexpression of miR-205 may represent a pejorative prognostic marker in DTC, and studies focusing on clinical outcome are required for a better definition. In all the mentioned studies, prognostic effect of miRNAs was based on the evaluation of the expression in tumour tissue. Importantly, tumour-derived miRNAs are also released into the bloodstream (Mitchell *et al.* 2008), where they can be detected and therefore used as circulating biomarkers. Although reliability and accuracy of circulating miRNAs as tumour markers are limited by the possible discordant distribution between tissue and the bloodstream (Garcia *et al.* 2008, Heegaard *et al.* 2012), they are considered promising diagnostic and prognostic tools in various types of cancers, such as lung, stomach and ovary neoplasms (Kroh *et al.* 2010, Tsujiura *et al.* 2010, Cheng *et al.* 2011, Shen *et al.* 2011). Role of circulating miRNAs as biomarkers is still under evaluation in thyroid cancer. Besides performing miRNAs evaluation on tumour tissues, the previously mentioned study by Lee and coworkers demonstrated that PTC-related miRNAs can be measured in plasma (Lee *et al.* 2013). Importantly, authors reported that miR-222 and miR-146b were overexpressed in plasma from patients with PTC compared with plasma from healthy individuals and that circulating levels significantly decreased after surgery. This suggests a close relationship between circulating miRNAs and active disease. To date, studies specifically assessing the feasibility of circulating miRNAs in the prognostic setting are missing, so their introduction into clinical practice is still far from reality.

Conclusions

To date, definition of genetic events leading to the development of cancer is possible in the vast majority of DTC patients. Translation of biological knowledge into clinical practice represents the next target to be achieved. In the recent years, the application of molecular characterisation is slowly but progressively entering into the diagnostic setting, with the aim to improve clinical management of patients harbouring thyroid nodules with indeterminate cytology. By contrast,

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current role of molecular analysis is extremely limited in the prognostic setting. Among the most frequent and deeply characterised molecular alterations related to DTC, $BRAF^{V600E}$ was the only mutation showing unequivocal prognostic effect. Nevertheless, the mutation demonstrated poor specificity, and therefore limited PPV, for the identification of patients with more aggressive disease, and was not eligible for being used as prognostic marker when it occurs separately. Further research allowing a strict definition of the possible heterogeneity of $BRAF^{V600E}$ in PTC and the deriving biological and clinical implications may allow us to overcome the limitations of the mutation in the prognostic setting. Indeed, a quantitative, rather than qualitative, analysis of the BRAF mutation may improve its specificity as prognostic marker. New molecular prognosticators with possible higher specificity for the identification of the subgroup of DTC patients with worst outcome are emerging due to the application of the novel NGS techniques. These include mutations of TERT promoter and TP53. Although the actual prognostic effect of TP53 alterations requires further evaluation, mutations of the TERT promoter have clearly demonstrated association with clinical outcome. Nevertheless, this genetic marker needs to be analysed according to a multigenetic model, as its prognostic effect becomes negligible when mutations are present in isolation. The co-occurrence of driving mutations is also emerging as an independent genetic signature of aggressiveness, but it needs further validation to be applied in clinical practice. To date, any DTC-related molecular alteration has demonstrated enough accuracy to be used in isolation in clinical practice. Therefore, current prognostication of DTC necessarily relies on a multivariable approach, possibly combining clinico-pathological and genetic characteristics. The cooperation between clinics and genetics was suggested by Xing and coworkers (2013) who demonstrated synergistic prognostic effect between BRAF mutation and several clinico-pathological features, including lymph node and distant metastasis, stage IV, and age at diagnosis. This approach has been adopted by the 'continuum of risk' model introduced by the latest ATA guidelines (Haugen *et al.* 2016), which includes both clinico-pathological factors and genetic features, particularly BRAF and TERT promoter mutations. To date, only somatic mutations have been assessed as prognostic markers. Nevertheless, tumour tissue may be unavailable and molecular characterisation of primary tumour may be misleading in metastatic patients. Although still based on preliminary data, use of BRAF mutation detected in circulating free DNA and circulating miRNAs as prognostic markers seems to be feasible. Nevertheless, a great effort is required to overcome the technical issues and refine prognostic effect, and their introduction into clinical practice is still far from reality.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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