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RESEARCH ARTICLE

MOLECULAR MARKERS AND THYROID CANCER.

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Abstract

Thyroid cancer is the most common malignant tumor of the endocrine system and includes well differentiated forms, namely papillary and follicular carcinomas, and the poorly differentiated and anaplastic carcinomas that result from the transformation of thyroid follicular cells. Notably, 5-10% of all thyroid cancers are medullary thyroid cancers that arise from parafollicular or C cells. The most common genetic mutations in papillary and follicular thyroid cancers are point mutations of the *BRAF* or *RAS* genes, while the most common chromosomal alterations are *RET/PTC* and *PAX8/PPARγ* rearrangements.The inherited form of medullary thyroid cancer is transmitted as an autosomal dominant trait due to a germline mutation of the *RET* proto-oncogene, but these mutations occur also in some sporadic cases. The most frequent initial manifestation of thyroid cancer is the appearance of a nodule. In almost all cases, these nodules are benign; in fact, less than 5% are malignant. However, some cases are misdiagnosed, and many patients are subjected to unnecessary surgery. Therefore,, therefore, it is important that patients undergo an accurate pre-surgery evaluation. The most reliable diagnostic test for thyroid nodules is fine needle aspiration cytology, which accurately distinguishes between a benign and malignant lesion in most cases. However, cytological discrimination between malignant and benign follicular cancer is often difficult because of poor quality samples. Here we review the various types of thyroid cancer, the associated point mutations and characteristic gene rearrangements.

Abbreviations:-

PTC, papillary thyroid cancer; FTC, follicular thyroid cancer; ATC, anaplastic thyroid cancer; MTC, medullary thyroid cancer; MEN;multiple endocrine neoplasia; FMTC, familial medullary thyroid carcinoma; MAPK, mitogen-activated protein kinase; PPFP, PAX8/PPARγ fusion protein

Types of thyroid cancer:-

Differentiated thyroid carcinomas:-

Thyroid cancer is the most common malignant tumor of the endocrine system (Gandhi et al., 2010)). Most thyroid cancers derive from thyroid follicular cells and include such well-differentiated forms as papillary and follicular carcinomas (PTC and FTC, respectively) and poorly differentiated forms such as anaplastic carcinoma (ATC). Papillary thyroid carcinoma is the main thyroid cancer and accounts for about 80% of all cancers. Papillary carcinomas are divided into: classic, follicular, solid, tall cell, oncocytic cell, and intestinal (Sherman, 2003). Papillary thyroid carcinoma is diagnosed based on its distinctive nuclear morphological features, which are common to all PTC variants. The nucleus of PTC cells has aground-glassappearancewithdepleted chromatin, nuclear membraneirregularities(the so-called coffee-bean grooves andnotches), and, on two-dimensional imaging,nucleoli-like cytoplasmic invaginations known as "pseudonucleoli". Tall-cell andintestinal PTCs havea poorer prognosis than

other PTCs. Papillary thyroid cancer is the most common thyroid tumor that develops after exposure to radiation (Dinets et al., 2012).

About 15% of all thyroid cancers are follicular thyroid cancers (FTC) (Cipriani et al, 2015). Follicular thyroid carcinoma is considered more aggressive than PTC. It occurs only rarely after radiation exposure and generally in a slightly older age group with respect to PTC. Moreover, FTCs are less common in children than in adults. Follicular carcinomas are divided into conventional and oncocytic (Hürthle cell) subtypes. Hürthle cell tumors are atypical neoplasms characterized by the presence of oncocytes (also called "Askanazy" or "oxyphilic" cells). These cells are large, and(delete) have hyperchromatic nuclei and an eosinophilic granular cytoplasm. Another characteristic of Hürthle cell cancers is the presence of numerous mitochondria in the cytoplasm. These carcinomas are more likely than FTC to be bilateral and multifocal, and tend to metastasize to lung and bone via the bloodstream (Cheung et al, 2000). Follicular carcinoma and its benign counterpart, follicular adenoma, share cytological features and differ from each other only by the invasion of tumor cells into the capsule or blood vessels. This distinction is rarely possible in cytology specimens and can be difficult even in tissue sections. Consequently, surgery to remove all or a large portion of the thyroid gland may be necessary to obtain sufficient tissue for a definitive diagnosis of FTC.

Poorly differentiated and anaplastic thyroid carcinomas:-

Poorly differentiatedthyroid tumors (2–3% of all thyroid cancers) are tumors that have a solid/trabecular/insular growth pattern, do not have the classic nuclear features of papillary carcinoma, and have a convoluted nucleus, necrosis or mitotic activity. Anaplastic thyroid carcinomas (1-2% of thyroid cancers) are extremely malignant. Both poorly differentiated and anaplastic thyroid carcinomas can arise *de novo* or from a preexisting well-differentiated PTC or FTC (Patel and Shaka, 2006). Anaplastic thyroid cancers have a very poor prognosis due to their aggressive behavior. They are more frequent in subjects living in iodine-deficient areas, and have a rapid course and early dissemination. The most common sites of distant spread are, in descending order, the lung, bone and brain. Anaplastic thyroid tumors do not respond to radioiodine therapy or TSH suppression with thyroxine unlike the other types of thyroid cancer. Surgical removal is the best treatment for ATC. Unfortunately, most patients present with a very advanced and large lesion, thus surgery is not feasible. Although surgery is not curative, patients will feel better if the lesion is removed.

Medullary thyroid carcinoma:-

Medullary thyroid carcinoma (MTC) arises from the parafollicular or C cells of the thyroid gland and accounts for approximately 5-10% of all thyroid cancers: 75% are sporadic and 25% are hereditary MTCs transmitted in an autosomal-dominant pattern (Chernock and Hagemann, 2015). Inherited MTCs present as multiple endocrine neoplasia (MEN) type 2A or 2B, or familial medullary thyroid carcinoma (FMTC). Genetic screening must be performed also in sporadic cases because about 4-5% of apparently sporadic cases of MTC are actually hereditary.

Gene mutations associated with thyroid carcinomas:-

The most common genetic mutations in PTC and FTC are point mutations that involve the *BRAF* and *RAS* genes, while the most common chromosomal rearrangements are *RET/PTC* and *PAX8/PPARγ*(Musholt, 2010; Ciampi, 2007; Xing. 2013). Many of these alterations involve the mitogen-activated protein kinase (MAPK) pathway. This intracellular cascade regulates cell growth, differentiation and response to growth factors, hormones and cytokines that interact with cell surface tyrosine kinase receptors, and affect apoptosis and cell survival. Mutation in *BRAF* or *RAS* occurs in about 70% of PTCs, suggesting that activation of the MAPK pathway is essential for the onset of carcinogenesis and that the alteration of a single effector is sufficient to cause cell transformation (Song et al., 2015). *BRAF* and*RAS* mutations are mutually exclusive (Celestino et al., 2012); however, multiple gene mutations were recently identified in several patients (Rossi et al., 2015). While *RET/PTC* rearrangements are particularly frequent in children (50-60%), *BRAF* point mutations are more common in adults than in children (50% vs 0-12%).

BRAF gene:-

The wild-type *BRAF* gene is localized on chromosome 7q24; it consists of 119 base pairs and encodes a cytosolic serine-threonine kinase. This protein belongs to the RAF family of proteins (A-RAF, B-RAF, and C-RAF), which are intracellular effectors of the MAPK signaling cascade. The physiological activation of RAS by various growth factors, hormones and cytokines activates RAF, which phosphorylates and activates MEK1 and MEK2 that, in turn, phosphorylate and activate ERK1 and ERK2. The latter phosphorylate other proteins, many of which are also kinases, thereby determining alterations in the expression of various genes (Tang and Lee, 2010). Of the three RAF proteins, BRAF is the one most often expressed in the thyroid and has higher basal kinase activity than either A- or

C-BRAF, and is also the most potent activator of MEK (Ciampi and Nikiforov, 2005). Among the more than 40 *BRAF* mutations identified so far, the most common variant is T1799A, which determines a substitution of a thymine with an adenine at amino acid level thereby resulting in a substitution of a valine with a glutamic acid at codon 600 of the protein (V600E). Inactive BRAF presents a bilobar structure caused by a hydrophobic interaction between residues of the activation loop (A loop) and residues of the P loop (which are ATP binding sites). This conformation cannot bind to ATP and, in the dephosphorylated wild-type protein, the hydrophobic interactions between the A and P loops maintain the protein in an inactive conformation. V600E and other mutations interrupt this conformation thereby resulting in a continuous activation form designated "catalytically competent" (Ciampi and Nikiforov, 2007). *BRAF* mutations have been implicated in tumorigenesis, dedifferentiation and increased tumor aggressiveness (Han et al., 2014).

In addition to PTCs, *BRAF* mutations were found also in poorly differentiated and ATCs insurgent on pre-existing PTC, especially in well-differentiated areas (Nikiforova, 2003; Quiros, 2005). *BRAF* mutations are found mainly in the tall cell variant of papillary carcinoma that has a notoriously poor prognosis, whereas they are rare in the FTC variant.

RET/PTC rearrangements:-

Other specific molecular markers of PTC are members of the *RET/PTC* chimeric gene family that constitutively induce receptor tyrosine kinase activity. The *RET* gene is located on chromosome 10q11.2, and encodes a 1,092 amino acid long protein, which is a transmembrane tyrosine-kinase receptor involved in the control of cell differentiation and proliferation (Elisei, 2001; Romei, 2012). RET consists of three domains: an extracellular ligand binding domain, a hydrophobic transmembrane domain and an intracellular tyrosine kinase domain. RET binds the family of GDNF (glial neuron derived factor) neurotrophic growth factors. In normal conditions, binding of the ligand to the extracellular portion of the receptor allows dimerization of the receptor, phosphorylation in tyrosine kinase cytoplasmic domain and consequent activation of the MAPK signaling pathway (Prescott, 2015).

*RET/PTC*is formed by the fusion of the 3' portion of the *RET*gene, which encodes the receptor tyrosine kinase, with the 5' portion of various unrelated genes. The two most common rearrangements, namely, *RET/PTC1*and *RET/PTC3*, are paracentric inversions since both *RET* and PTC and their respective fusion partners, *H4*and *NCOA4* (also known as *ELE1, RFG or ARA70*), reside on the long arm of chromosome 10. *RET/PTC2*and other more recently identified *RET/PTC* variants are all interchromosomal translocations (Jhiang, 2000). All fusions contain the intact tyrosine kinase domain of the RET receptor and enable the RET/PTC chimeric protein to activate the RAS-RAF-MAPK cascade and initiate tumorigenesis. *RET/PTC* variants are more frequent in patients with a history of radiation exposure (50–80%) and in PTCs in children and young people (40–70%) than in adults. *RET/PTC1* is the most common *RET/PTC* rearrangement and is found in up to 60–70% of PTC cases, whereas *RET/PTC3* accounts for 20–30%, and other novel rearrangements for less than 5%. *BRAF* and *RET* oncogenic alterations appear to be mutually exclusive but also in this case, a recent study identified the simultaneous presence of these genes (Rossi et al., 2015).

RAS genes:-

Other genes involved in thyroid malignancies belong to the *RAS* family. Point mutations of the *RAS* genes are not restricted to a particular type of thyroid tumor, and indeed are found in FTCs, PTCs and follicular adenomas. However, the spectrum of DNA alterations differ between FTC and PTC. In particular, mutations of the *HRAS* and *NRAS*genes have been observed in FTCs and in follicular adenomas, but rarelyin PTCs. The *RAS* genes (*HRAS, KRAS*, and *NRAS*) (Gupta, 2013; Lai, 2015)are implicated in the regulation of cell proliferation and differentiation, in coupling membrane receptors and in the intracellular signal transduction machinery. The tumorigenic potential of the *RAS* genes is activated by point mutations in codons 12, 13, 61 that can block p21 in a constitutively activated form that is unable to hydrolyze the GTP linked to it, thereby stimulating cell proliferation uncontrollably. The point mutations in codons 12,13 and 61 affect all three *RAS* genes and are found in 50% of follicular adenomas and carcinomas (Xing, 2013).

Recent reports (Moura et al., 2015) have identified *RAS* mutations, particularly *HRAS* and *KRAS*, in 0-43% of sporadic MTCs usually in wild-type RET cancer, which suggests that RAS activation is an alternative genetic event in sporadic MTC. Assessment of *RAS* mutation status can help to define therapeutic strategies in *RET* wild-type MTC. Medullary thyroid cancer patients with *RAS* mutations have an intermediate risk of aggressive cancer, i.e.,

between those with *RET* mutations in exons 15 and 16, which are associated with the worst prognosis, and cases with other *RET* mutations, which have the most indolent course.

PAX8-PPARg rearrangements:-

Rearrangements of the *PAX8-PPARγ* gene have been found in 4%–13% of follicular adenomas. *PAX8-PPARγ* rearrangements and *RAS* mutations account for about 75% of all FTCs and seem to be mutually exclusive thereby supporting the hypothesis that there are distinct molecular subtypes (McHenry and Phitayakorn, 2011).*PAX8* (paired box 8) is a gene that encodes the thyroid-specific paired domain transcription factor essential for the differentiation of follicular cells and the regulation of thyroid-specific genes.*PAX8* has 12 exons; alternative splicing of exons 8-10 results in the production of multiple protein isoforms. *PPARγ* (peroxisome proliferator–activated receptor) belongs to the nuclear receptor family of transcription factors that regulates cell differentiation and lipid metabolism. In fact, the protein is the major regulator of adipogenesis and a potent modulator of whole-body lipid metabolism and insulin sensitivity (Nikiforov, 2008). These two genes can fuse with translocation t(2; 3)(q13;p25)to form a new fusion gene that expresses a PAX8/PPARγ fusion protein, designated PPFP. Typically, the translocation fuses *PAX8* intron 10 with the intron immediately preceding the first coding exon of *PPARγ*. Alternative splicing creates multiple RNA isoforms within the same tumor. Specifically, transcripts with PAX8 exons 1-8, 1-9 and 1-10 with exon 9 deleted have been detected fused to the first coding exon of PPARγ, and all these maintain the *PPARγ* reading frame (Raman and Koenig, 2014)). The PAX8/PPARγ fusion protein has been implicated in thyroid follicular oncogenesis because it abrogated normal PPARγ function (Marques et al., 2002). The oncogenic effects of PPARγ could be related to constitutive overexpression of the full length PPARγ domain, interference with wild-type PPARγ function, alterations of PAX8 function, formation of novel fusion gene activities, or a combination of these events (Kroll et al., 2000). PPFP appeared to affect several key aspects of tumorigenesis by increasing cell cycle transition, reducing apoptosis and inducing loss of both anchorage dependence and contact inhibition (Placzkowski et al., 2008). Each of these effects is a facet of malignant transformation and together support the concept that PPFP interferes with multiple growth-regulatory pathways (Gregory Powell et al., 2004).

The *PAX8/PPARγ* fusion oncogene has been identified only in follicular adenomas and carcinomas, which supports the concept that papillary and follicular tumors have distinct genetic evolutions, which may explain the phenotypic and clinical differences between these two tumor histotypes. The protein product of *PAX8/PPARγ*acts, at least in part, by inhibiting wild-type PPARγsignaling thereby suggesting that such tumors could be sensitive to PPARγagonist therapy (Algeciras-Schimnich et al.,2010). Chromosomal alterations of *PPARγ* that result in the expression of the fusion protein PPFP may be an early event in the development or progression of FTC. In addition, the *PAX8/PPARγ* rearrangement may not be sufficient for the development of a malignant phenotype, and additional genetic or epigenetic events may be required to enable the full phenotypic expression of FTC (Cheung et al., 2003). Tumors harboring *PAX8/PPARγ* tend to occur at a young age, be small, have a solid nested growth pattern, and to more frequently be vascular (Şahpaz et al., 2015). The PAX8/PPARγ rearrangement is also found in a small fraction (2–10%) of follicular adenomas.

RET point mutations:-

The inherited form of MTC is transmitted as an autosomal dominant trait due to a germline mutation of the *RET* proto-oncogene that encodes a receptor tyrosine kinase (Yeganeh et al., 2015). The disease may manifest clinically as MTC or as part of syndrome involving other multiple endocrine glands (MEN2) (Eng et al., 1996). The molecular pathology of inherited MTC is constitutive activation of the proto-oncogene *RET*. Several independent mutations in the extracellular and tyrosine kinase domainsof the *RET* gene are pathognomonic of MEN 2A, MEN 2B and FMTC (Mulligan, 2014). Germline *RET* mutations are of diagnostic significance, and useful in the treatment of MTC patients and in first-degree relatives of MEN patients, who have 50% risk of inheriting the mutated gene. In fact, genetic screening can reveal carriers of the mutated gene among relatives of an affected individual who are destined to develop MTC. Consequently, these carriers can undergo surgery or timely preventive treatment. In general, *RET* testing is a mandatory tool in the clinical assessment of sporadic or apparently sporadic MTCs, and of MEN2 carriers. In addition, the risk of developing aggressive cancer can be assessed based on the type of *RET* mutation. The importance of *RET* testing emerges from the finding that the efficiency of RET inhibitors against MTC is associated with the type of *RET* mutation (Schulten et al., 2011). Exome sequencing data indicate that mutations in *RET*, *HRAS*, and *KRAS*, are the only recurrent driver mutations present in MTC (Moura et al., 2015).

Thyroid nodules:-

The most frequent initial manifestation of thyroid cancer is the appearance of a nodule. Thyroid nodules are very common in the general population and in most cases (95%) they are simply hyperplasic or benign lesions. Therefore, given the low mortality rate linked to thyroid cancer and the small percentage of malignant thyroid nodules, an accurate diagnosis is necessary to spare patients unnecessary surgery. The most reliable diagnostic test for thyroid nodules is fine needle aspiration (FNA) cytology, which establishes the diagnosis of a benign or a malignant lesion in most cases, but cytological discrimination between malignant and benign follicular neoplasms remains difficult. Moreover, PTC, MTC and ATC can be easily diagnosed by FNA cytology thanks to their specific histological characteristics. However, many thyroid FNAs are "indeterminate". In such cases, FNA sampling is usually repeated or even surgery is performed in the attempt to establish a diagnosis, and these procedures result in additional morbidity and higher health care costs. Moreover, patients with malignant tumors and indeterminate FNA cytology typically undergo limited surgery, *i.e.* lobectomy, and once malignancy is established by pathological examination of the excised nodule, these patients must undergo a second operation to complete the thyroidectomy, which is also associated with additional costs and morbidity. In addition, 1–3% of nodules diagnosed as benign on FNA cytology are subsequently found to be malignant, and the delay in treatment places patients at risk of disease progression.

Fine needle aspiration and molecular analysis:-

The American Thyroid Association Guidelines (Haugen, 2016; Wells, 2015) acknowledge the advantages of molecular markers in thyroid cancer management. Some new molecules have helped to shed light on the mechanisms underlying thyroid cancer but most of them are poorly informative. In fact, as shown in Alonso-Gordoa et al (2015), testing for *BRAF*, *H-N-Kras* and *RET/PTC1* and *RET/PTC3* rearrangements revealed that about 90% of PTCs are due to *BRAF-RAS* mutations and *RET* rearrangements, 85% of follicular cancers are due to *RAS* mutations and *PAX8/PPARγ* rearrangements, and 60% of poorly differentiated and anaplastic cancers are due to *BRAF* and *RAS* mutations. *RET* point mutation testing identifies about 95% of hereditary MTCs and testing for *RET* point mutations combined with *RAS*mutations identifies about 60% of sporadic MTCs. Figure 1 shows the more frequent thyroid genetic alterations and their main pathological associations.

Molecular testing for *BRAF*, *RAS*, *PAX8-PPARγ* and *RET-PTC* has improved the management of patients with thyroid nodules (Mehta, 2013; Yip, 2014; Witt, 2013)and of patients with indeterminate FNA cytology (Figure 2).

Figure 1:-Genetic alterations and thyroid pathology.

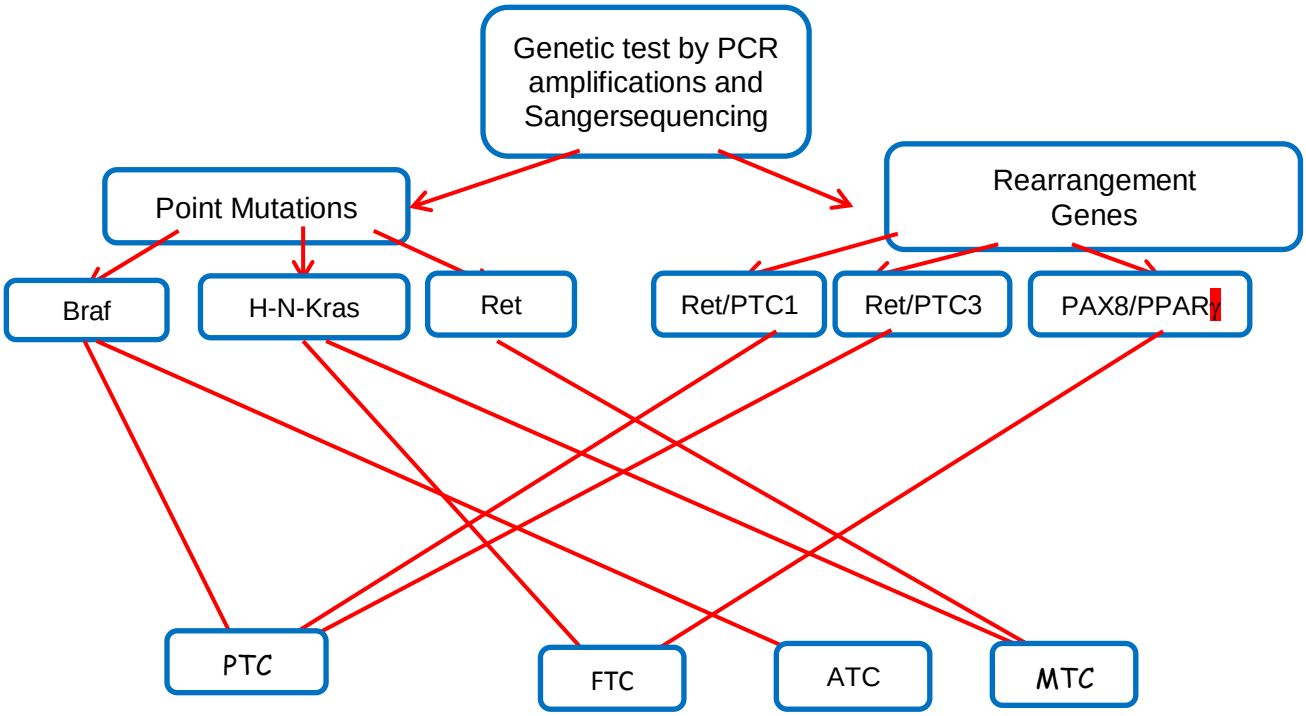
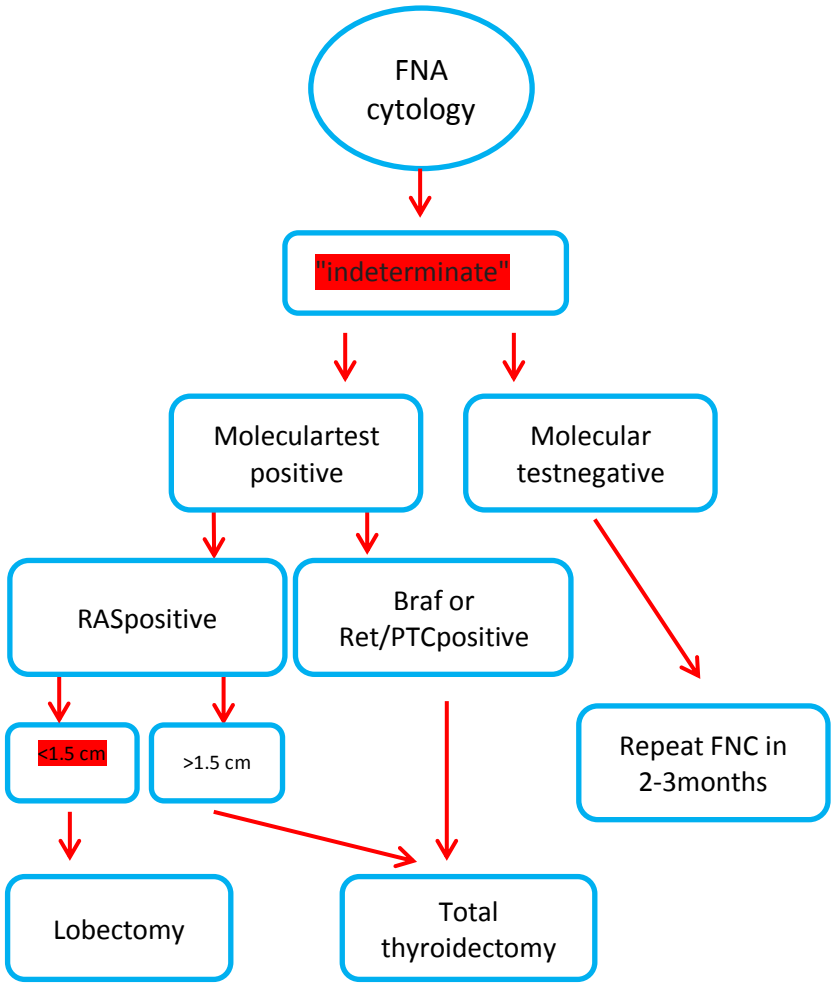


Figure 2:-Molecular diagnosis and "indeterminate" FNA cytology.



Conclusions:-

Fine needle aspiration cytology is the most reliable procedure with which to obtain a preoperative diagnosis of differentiated thyroid carcinoma provided that problems related to poor samples are overcome. The study of genetic modifications has increased the diagnostic accuracy of traditional cytology, and the use of FNC washer to reveal a molecular signature of the lesion means that almost all the cellular material can be used for the cytological examination.

An understanding of the alterations in different molecular pathways of thyroid cancer is a prerequisite for the development of new targeted drugs. Thanks to genetic testing, treatment can be customized for each patient even in inconclusive cases, and surgery can be reserved for malignant cases with a consequent substantial saving in terms of health care costs. In fact, patients with an inconclusive diagnosis often repeat nodule sampling or undergo limited surgery i.e., lobotomy. If a diagnosis is established, these patients may require a second operation to complete the thyroidectomy, thereby increasing costs and patient discomfort.

The molecular genetic study of oncogenes involved in thyroid cancer can help to formulate a more accurate diagnosis (Nikiforova and Nikiforov, 2009). In recent years, thanks to the progressive knowledge of the molecular mechanisms involved in thyroid pathology together with the increasing use of molecular biology techniques in cytopathology, some molecular methods are now widely used in routine diagnostics (Nikiforov et al., 2013). In fact, molecular testing of FNA samples significantly improves the accuracy of the cytological diagnosis of thyroid

nodules, and therefore, methods that improve the sensitivity and specificity of FNA cytological diagnosis are highly desirable and could have a significant impact on clinical care (Cantara, 2010; Ohori, 2013; Valderrabano, 2015).Lastly, the unraveling of the genetic mechanisms and the identification of molecular alterations involved in thyroid pathology have resulted in a rationale for the management and use of new therapeutic agents that have changed and will continue to change the treatment of thyroid cancer.

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