Aus dem Institut für Pathologie
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DISSERTATION

Yes-associated protein (YAP) expression and its biological role in thyroid gland

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1 Introduction

1.1 Yes-associated protein

Yes associated protein (YAP) was first identified in 1994 as a novel chicken protein of 65 kDa molecular weight by its interaction with SH3-domain of the product of proto-oncogene *c-yes* (c-YES protein), a member of non-receptor tyrosine kinase SRC-proteins family, and for this it was named YAP65 (Sudol 1994). One year later, the human *yap*-gene sequence was mapped to the chromosome band 11q13 centromeric to the *ccnd/bcl 1* locus. At the same time, YAP mRNA expression profile was detected in all adult human tissues except peripheral leukocytes with relatively high levels of message in placenta, prostate, testis, ovary and small intestine (Sudol et al. 1995).

1.1.1 YAP molecular structure

YAP has a characteristic molecular structure by harboring several binding motifs that mediate its interactions with their partner proteins (Figure 1-1).

WW domain is a protein-protein interaction module that recognizes short proline-rich motif (PY) of diverse proteins which involved in various signalling pathways. The name refers to two signature tryptophan (WW) residues that are spaced 20-22 amino acids apart. There are four well known classes of WW-domains, distinguishable by the amino acid sequence of the proline-rich motifs (PY), that they recognize on the target protein. YAP's WW-domain belongs to class I which recognizes the PY motif (PPxY) (Sudol and Hunter 2000). YAP-protein has two isoforms resulting from *yap* gene splicing: a short form (YAP1) that possesses only one WW-domain and a long form (YAP2) that has two WW- domains (Sudol et al. 1995). WW-domain mediates the majority of protein-protein interactions described to date for YAP protein.

Coiled coil motif is a structural motif, in which 2-7 alpha-helices are coiled together. Many coiled coil type proteins are involved in important biological functions such as the regulation of gene expression (Liu et al. 2006).

PDZ binding motif **(**post-synaptic density, discs large, zonula occludens-1) is located at the COOH-terminus of YAP-protein. It mediates YAP's interaction with PDZ containing proteins like EBP50 (ERM-binding protein 50 kDa) at the apical membrane of epithelial cells (Mohler et al. 1999).

SH3-binding motif is a proline- rich motif that mediates YAP's binding to the SH3-domain of c-YES and other members of SRC-family (c.SRC) as well as of other signalling molecules containing SH3 domain like CRK (a member of non-receptor tyrosine kinase family SRC) and NCK (non-catalytic region of tyrosine kinase adaptor protein 1) (Sudol et al. 1995).

Figure 1-1: Domain organization of YAP-protein. It contains a WW domain (YAP1) or tow WW domains (YAP2), a SH3-binding motif, a predicted coiled coil (CC), a COOH-terminal PDZ interaction motif and a proline-rich at the N-terminus (Kanai et al. 2000).

1.1.2 Role of YAP-protein in human cells

Initially and because of its association with c-YES protein, it was thought that YAP-protein functions as a modular adaptor protein by regulating transduction of apical signals from the pathways that control ion transport, cytoskeletal organization, or gene expression in human epithelial cells (Mohler et al. 1999). Later, it was found that YAP-protein has a role in coactivation of gene transcription, where it binds to the PY-motif of PEBP2α through its WW-domain. Then fusing of the C-terminal region of YAP-protein to the DNA-binding domain of GAL4 resulted in a transactivation as strong as that of GAL4-VP16. These results indicated that PY-motif of a transcription factor represents a novel transcription activation domain that functions by recruiting YAP-protein to act as a strong transcription activator to the target genes (Yagi et al. 1999). Additional studies demonstrated that PY-motif presents in a broad range of transcription factors that mediate different physiological or pathological functions in human cells and tissues. Accordingly, now it is well known that YAP-protein can mediate different physiological and pathological processes in human tissues, and in each one it may play a different biological role.

1.1.2.1 Apoptotic function of YAP

P73 is one of the most important partners of YAP-protein through which can YAP-protein mediates its proapoptotic function in human cells. P73 is a transcription factor which belongs to the p53 family, along with p53 and p63. P73 and p63 mimic p53 function in human cells including transactivating many p53-target genes mediating tumour- suppressing and/or inducing apoptosis (Lohrum and Vousden 2000). Structurally, both of them (i.e., p73 and p63), express multiple isoforms as a result of alternative splicing of their genes, where there are 2 isoforms for each regarding the presence or absence of transactivation domain (TA-domain) that resulted from using two different promoters P1 or P2: the full-length protein isoforms that contain TA-domain (TAp73 and TAp63) and the NH-terminally truncated protein isoforms that missed TA-domain (ΔNp73 and ΔNp63). Additional complexity to this network of protein isoforms arises from multiple splicing of the COOH-terminus, by skipping of one or several exons. Thus far, nine transcripts were found for TAp73: α, β, γ, δ, ε, ζ, η, η_1 and Φ. Functionally, TAp73 and TAp63 mimic p53 function, whereas ΔNp73 and ΔNp63 act as dominant negative inhibitors of themselves and of other family members (Moll and Salde 2004).

1.1.2.1.1 YAP-dependent p73-mediated apoptosis in response to DNA-damage.

In response to DNA damage, the protein kinase c-ABL is activated to phosphorylate both of p73 at tyrosine 99 and YAP-protein at tyrosine 357 residue. This results in a dual effect. One the one hand, the phosphorylated YAP (Tyr 357) will stabilize p73 by binding with the PY-motif of p73 through its WW-domain. In this way it competes with E3 ubiquitin ligase itchy homolog (ITCH), an enzyme that induces proteosome-mediated p73 degradation (Levy et al. 2007). On the other hand, the phosphorylated YAP (Tyr 357) will translocate into the nucleus in a p73-dependent manner. In the nucleus, YAP assembles p73 to complex with PML and p300 proteins, and directs the protein complex to associate with p53AIP1 that in turn regulates the apoptotic response (Figure 1-2) (Strano et al. 2005). In this complex, PML plays an important role in stabilization of YAP by sumoylation of YAP-protein at Lysine 97 and 242 residues which in turn protects YAP-protein from proteosome-mediated degradation (Lapi et al. 2008).

YAP-dependent p73-mediated apoptosis induced by c-ABL activation is attenuated and negatively regulated by the pro-survival protein AKT. AKT has been reported to control the activity of a number of cellular proteins by promoting their phosphorylation to create at the site of phosphorylation a novel binding motif to 14-3-3 proteins. AKT mediates phosphorylation of YAP-protein at the Ser-127, und upon this phosphorylation 14.3.3 proteins are recruited to bind the phosphorylated YAP (Ser-127) which in turn promotes cytoplasmic localization of YAP-protein. As a result, YAP-protein will lose its tumour suppressor function in coactivation of p73 in the nucleus (Basu et al. 2003).

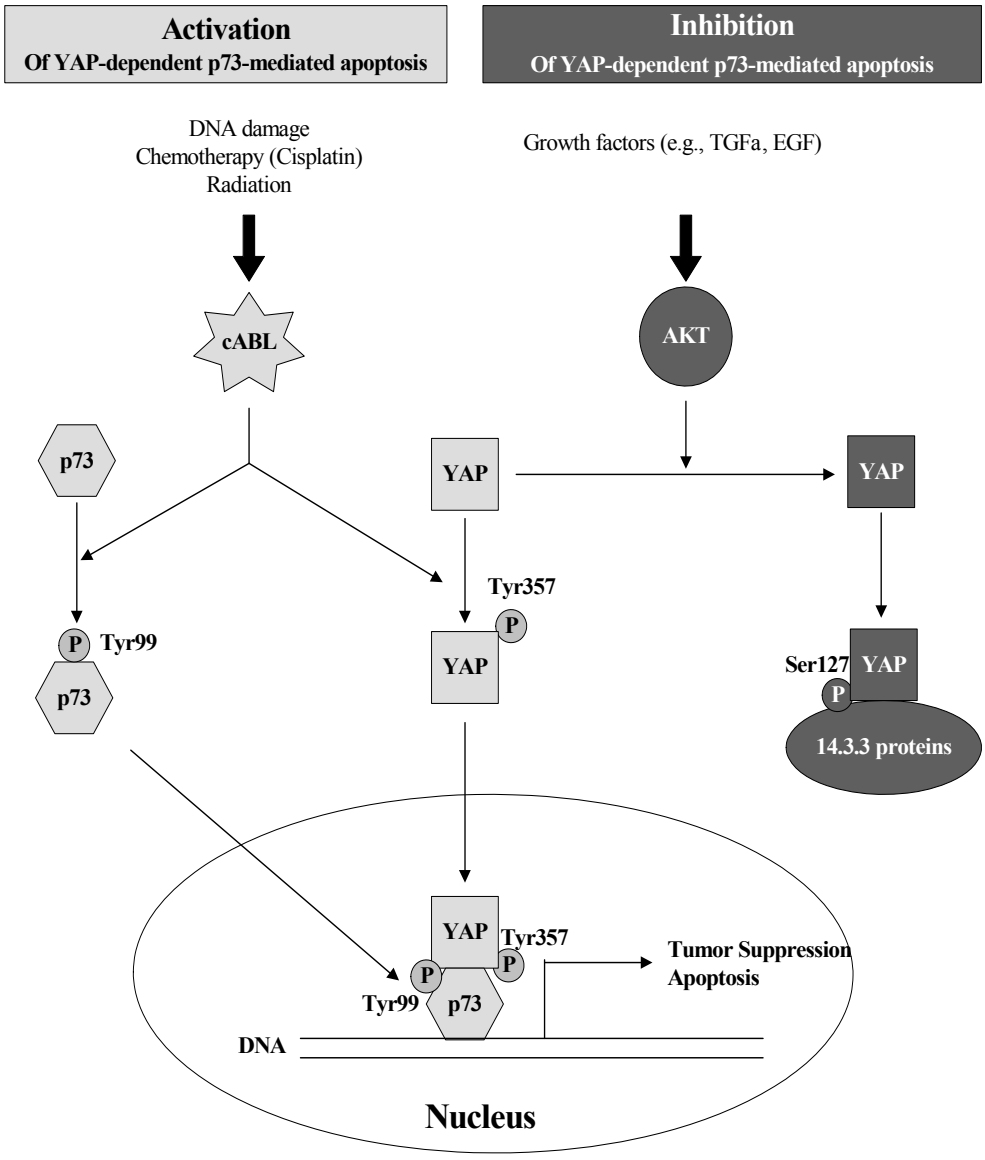

Figure 1-2: YAP-dependent p73-mediated apoptosis in response to DNA damage and its negative regulatory inhibition by AKT activation. DNA-damage induces c-ABL activation that promotes nuclear translocation of YAP to complex p73 and induces cell apoptosis. Oncogenic stimuli activate AKT to phosphorylate YAP and mediate its cytoplasmic sequestration by 14.3.3 protein family (Strano et al. 2005, Basu et al. 2003).

1.1.2.1.2 YAP-dependent p73-mediated apoptosis in response to FAS receptor stimulation

It has been found that YAP-protein represents the downstream of another apoptotic signalling pathway, the FAS/FASL signalling pathway (figure 1-3). In response to apoptotic stimuli, FAS receptors are stimulated to induce apoptosis in RASSF1- dependent manner. RASSF1 is a tumour suppressor protein that can be inhibited under oncogenic stimuli by a proto-oncogene RAF1, which binds to the RASSF1 activator, the tumour suppressor protein MST2, preventing its activation and triggering its apoptotic signalling (Matallanas et al. 2007). .

FAS stimulation by apoptotic stimuli resulted in activation of RASSF1 that starts to induce apoptosis by mediating disruption of the inhibitory RAf1-MST2 complex and assembling with MST2. Assembling of RASSF1 with MST2 resulted from activation of the latter to phosphorylate subsequently YAP-sequestering kinase, LATS1. LATS1 phosphorylation results in releasing the transcriptional regulator YAP and allowing it to translocate into the nucleus where it complexes and coactivates p73 to induce transcription of the proapoptotic target gene *puma* (Matallanas et al. 2007).

These findings are in complete contradiction to what is well known about MST-LATS-YAP cooperation as integral components of another tumour suppressor pathway, the Hippo pathway. This pathway considers YAP-protein as an oncogenic protein, and it inhibits YAP's oncogenic function by phosphorylation of LATS1/2, that in turn maintains cytoplasmic sequestration of YAP-protein by 14.3.3 proteins after its phosphorylation at Ser-127. Recently, this controversy about the cooperation of YAP-LATS in these tow different pathways was resolved, where it was demonstrated that RASSF1A can induce YAP-dependent p73-mediated apoptosis through activation of the tumour suppressors MST1/2 and LATS1/2 independently from Hippo pathway (Donninger et al. 2011).

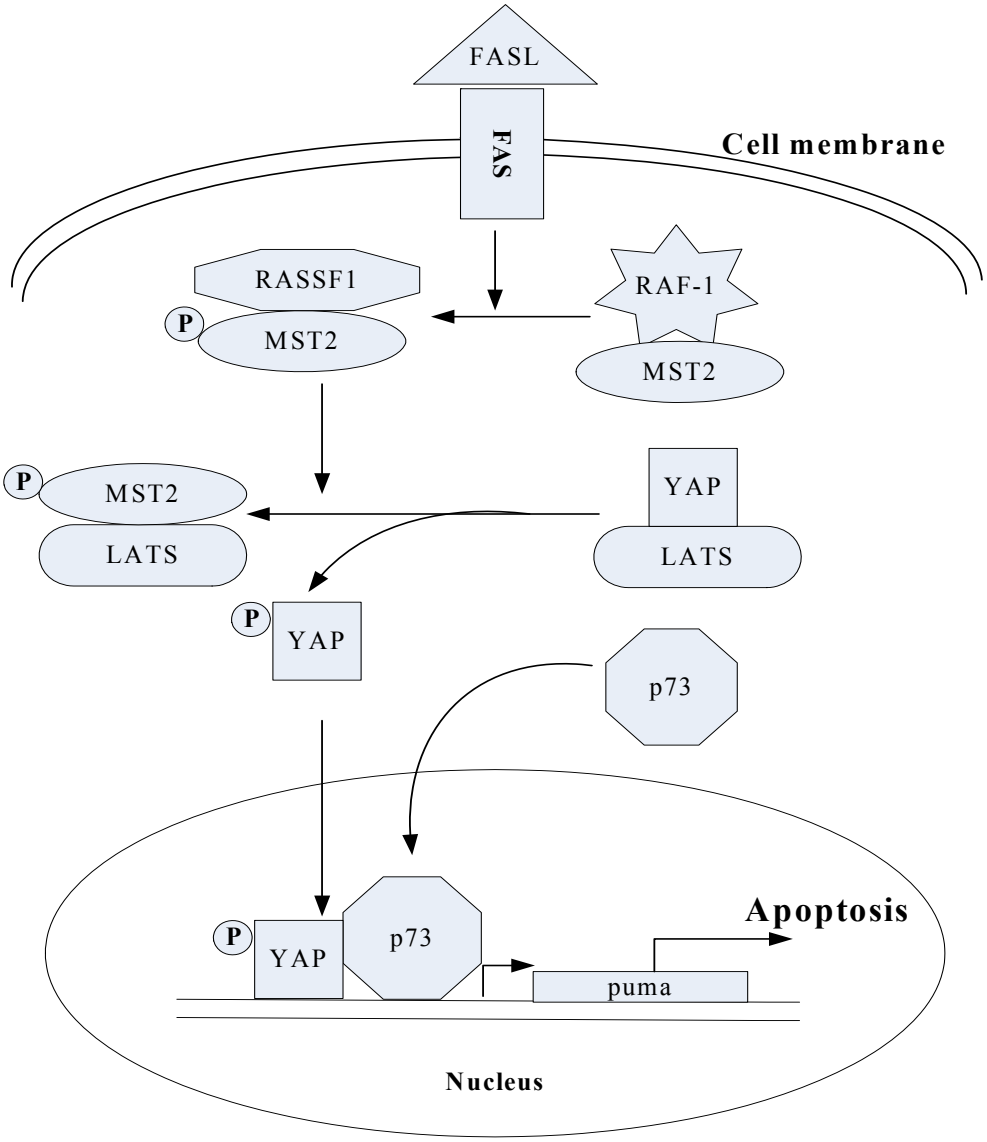

Figure 1-3: YAP-dependent p73-mediated apoptosis in response to FAS receptor stimulation. FAS receptor activation inhibits the oncogenic function of RAF-1 by releasing the tumour suppressor MST2 to activate the release of YAP from its cytoplasmic sequestration by LATS and translocates to the nucleus to induce p73-dependent puma-mediated apoptosis (Matallanas et al. 2007).

1.1.2.2 Oncogenic function of YAP

Recently, emerging evidence suggests YAP-protein as a candidate oncogene, where it exerts a function of potent transcription coactivator to induce oncogenic cellular processes: growth factor-independent cell proliferation, suppression of apoptosis and epithelial to mesenchymal transformation (EMT) (Overholtzer et al. 2006, Zender et al. 2006). The TEAD transcription factor is considered to be the main partner of YAP-protein by which it can exerts its oncogenic function (Zhao et al. 2008).

TEAD (TEA domain)-proteins represent a family of four transcription factors that share in common TEA DNA-binding domain. At least one *tead* gene is expressed in most of human adult tissues (Jacquemin et al. 1996). TEAD- dependent transcription by any one of the four TEAD proteins requires a transcriptional coactivation of YAP-protein (Vassilev et al. 2001). Surprisingly, although TEAD is concentrated in the nucleus, YAP is concentrated in the cytoplasm where most of it is either associated with 14.3.3 proteins, a family of proteins involved in transporting its binding partner from the nucleus to the cytoplasm (Fu et al. 2000), or bound to SRC/YES protein tyrosine kinase, the components of signal transduction pathways (Sudol 1994). However, in response to mitogenic stimuli, YAP-protein will be released from its sequestration by 14.3.3 proteins family into the cytoplasm and then it can be transported freely into or out of the nucleus, where it forms a transcriptionally active complex with TEAD (Figure 1-4) (Vassilev et al. 2001).

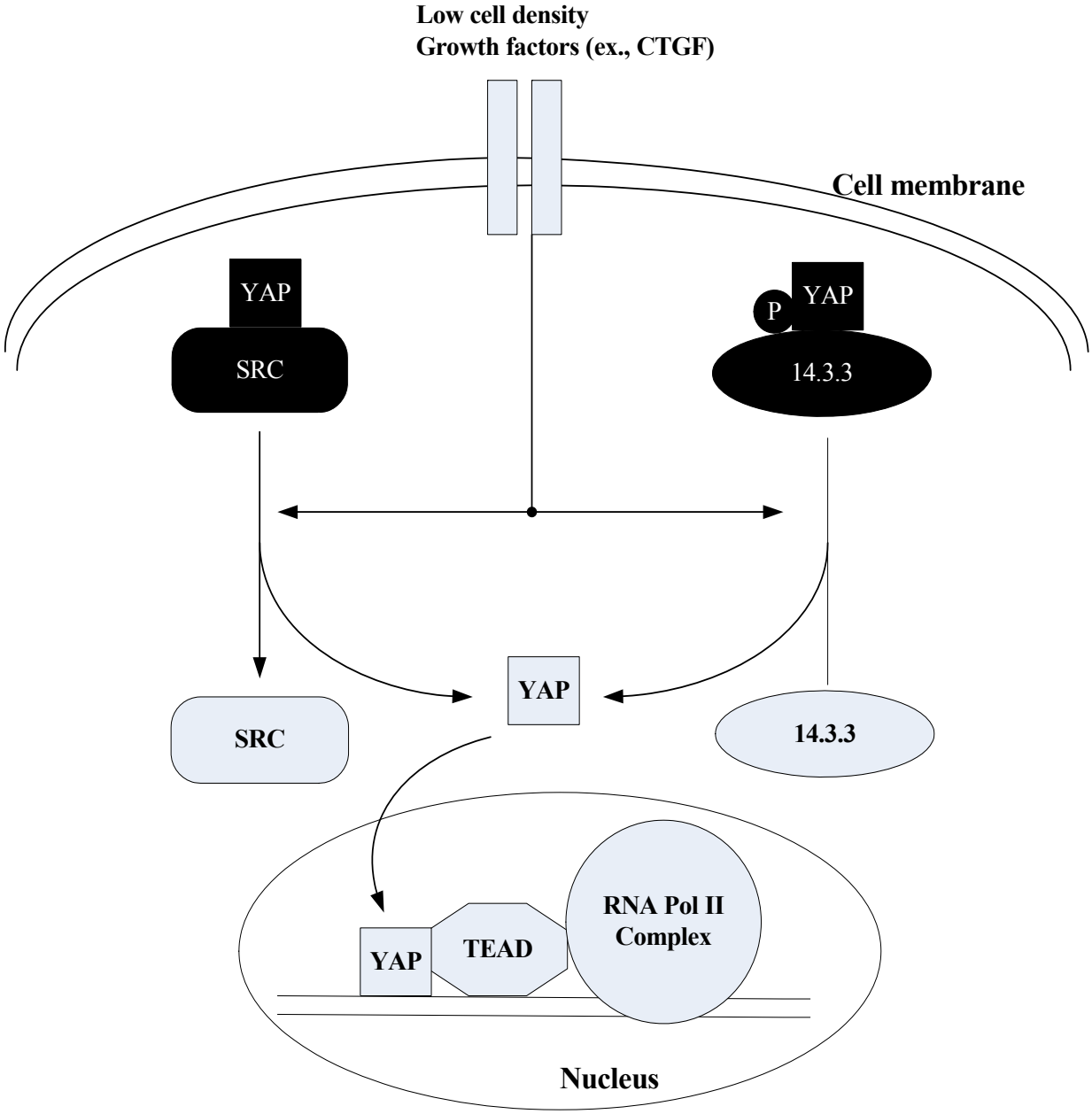

Figure 1-4: TEAD and YAP interaction in response to mitogenic stimuli. Mitogenic stimuli induce the release of cytoplasmic YAP-protein from its sequestration with 14-3-3 or Src-YES to translocate to the nucleus where it promotes TEAD-mediated oncogenesis (Vassilev et al. 2001).

YAP-dependent TEAD-mediated oncogenic function is negatively regulated by a potent tumour suppressor signalling pathway, the Hippo pathway. The Hippo pathway was initially identified in the fly *Drosophila melanogaster* to control organ size. Later, its core components are evolutionary conserved in mammals, as well as an essential role for this pathway was clearly established in regulating cell contact inhibition, organ size control and cancer development in mammals (Camargo et al. 2007, Dong et al. 2007, Zhao et al. 2007).

The most upstream of this pathway in mammals is WW45 (WW domain containing protein, 45 kDa molecular mass) (Figure 1-5). The remaining downstream components are represented by two closely related serine/threonine kinases MST1 and MST2 (Mammalian Ste20- like protein 1 and 2 kinases, respectively), and LATS1 and LATS2 (Large tumour suppressor kinases 1 and 2 respectively). The downstream effecter of this pathway is YAP-protein (Zhao et al. 2009).

Activation of this pathway is started by activation of the upstream WW45 to phosphorylate the cascade kinases MST1/2 and then LATS1/2. At the end of the cascade, the phosphorylated LATS1/2 will phosphorylate YAP-protein at the Ser-127 to create an interaction motif to 14.3.3 protein family and this will result in turn in retaining the oncogenic YAP-protein in the cytoplasm and aborting its action in inducing TEAD-mediated oncogenesis (Dong et al. 2007; Zhao et al. 2007).

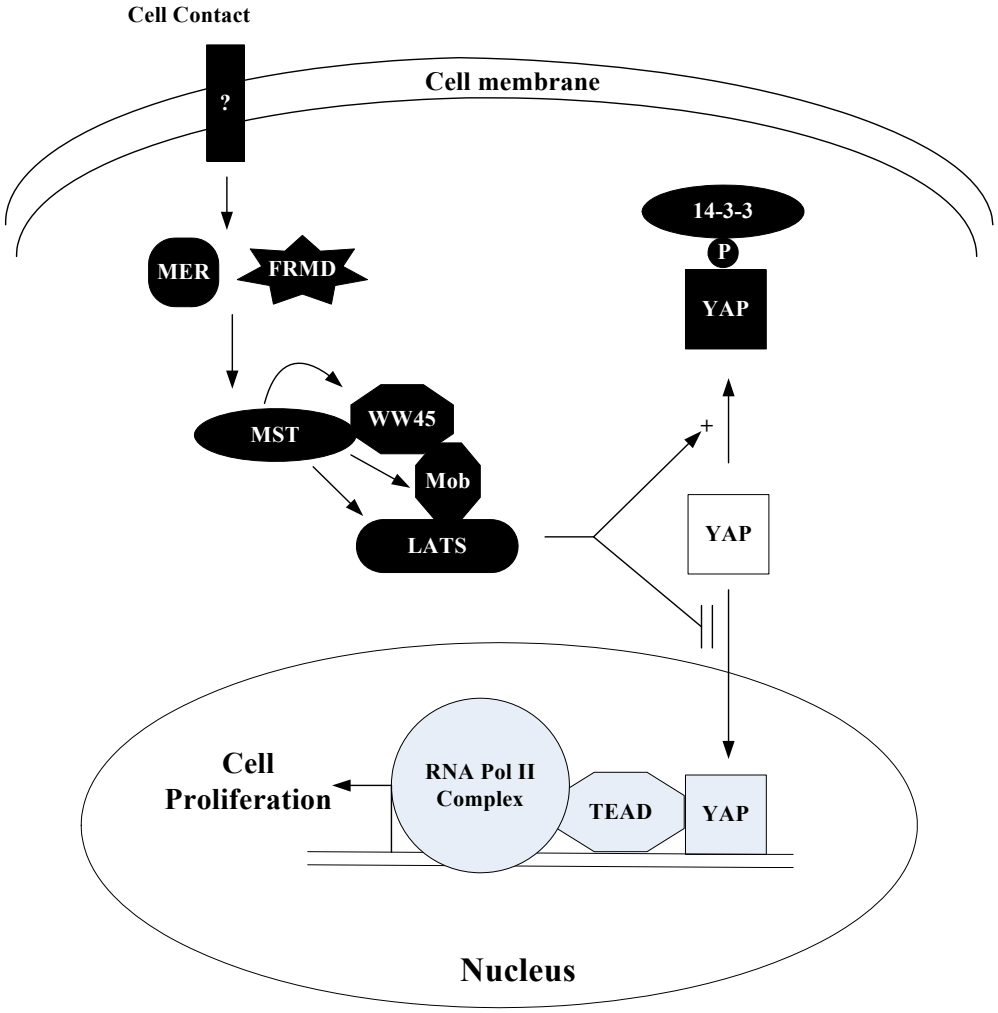

Figure 1-5: Hippo pathway in mammals. Activation of the cascade pathway is started by WW-45 and ended by phosphorylation of its downstream effecter YAP-protein that promotes its cytoplasmic sequestration in 14.3.3-mediated binding (Zhao et al. 2009).

1.2 Thyroid cancer

The clinically recognized thyroid cancer is regarded as a relatively rare human malignancy, where it accounts approximately only for 1% of all human malignancies in developed countries with an estimated annual incidence of 122,000 cases world wide (DeLellis and Williams 2004). However, thyroid cancer represents the most common malignancy in the endocrine system, and it is responsible for more deaths than all other endocrine cancers combined (Görges 2005). In addition, its incidence has steadily increased, and has almost tripled over the past 30 year wide world (Davies and Welch 2006, Albores- Saavedra et al. 2007, Colonna et al. 2007).

1.2.1 Classification

According to the (WHO) classification system of thyroid tumours (Table 1-1), primary thyroid tumors are classified as epithelial and non-epithelial, benign or malignant, with a separate category for lymphomas and miscellaneous neoplasms (Hedinger et al. 1988). In general, more than 95% of thyroid cancers are derived from thyroid follicular cells, while the remainder (less than 5%) is derived from C-cells, medullary carcinoma. Follicular cell- derived thyroid cancers are divided according to the degree of differentiation into 3 main histotypes: papillary carcinoma, follicular carcinoma and both of them are referred to as well differentiated follicular cell-derived thyroid cancers; and anaplastic carcinoma, which is referred to as undifferentiated follicular cell-derived thyroid carcinoma (Kumar et al. 2010).

Table 1-1: The (WHO) histological classification of thyroid tumours (Heidinger et al. 1988).

I. Epithelial Tumours
- A. Benign
  - 1. Follicular adenoma
- B. Malignant
  - 1. Follicular carcinoma
  - 2. Papillary carcinoma
  - 3. Undifferentiated (anaplastic) carcinoma
  - 4. Medullary carcinoma
  - C. Others

II. Non- epithelial Tumors
- A. Benign
- B. Malignant

III. Malignant lymphomas
IV. Miscellaneous

1.2.2 Pathogenesis of thyroid cancer

Thyroid carcinogenesis is a complex process where its initiation and progression occurs through a gradual accumulation of various genetic and epigenetic alterations, including activating and inactivating somatic mutations, alterations in gene expression patterns, micro-RNA (miRNA) dysregulation and aberrant gene methylation. However, all these alterations are clustered along four major signalling pathways: RET/RAS/BRAF/MAKP; TRK/PI3K/AKT; PAX8/PPARγ and MDM/p53/PTEN pathways (Figure 1-6) (Nikiforov and Nikiforova 2011; Kouniavsky and Zieger 2010).

Over the past years, the application of molecular technologies for studying thyroid carcinogenesis has elucidated critical genetic alterations in these pathways associated with the development of specific thyroid tumour histotype (Figure 1-6). In papillary carcinoma pathogenesis, cancer genes acting through the MAP kinase pathway, mainly BRAF and RAS mutation, RET/PTC rearrangement, have been clearly implicated. On the other hand, genes acting through the phosphatidylinositol 3-kinase (PI3K) pathway, namely PI3KCA and PTEN mutations, are implicated in the pathogenesis of follicular carcinoma. Finally, deregulation of the p53 pathway seems to be an important second step leading to the formation of poorly or undifferentiated carcinoma (Riesco- Eizaguirre and Santisteban 2007a). Similarly, germ line and somatic mutations of the RET oncogene are implicated in the pathogenesis of the familial and sporadic medullary carcinomas, respectively (Delellis & Welch 2006).

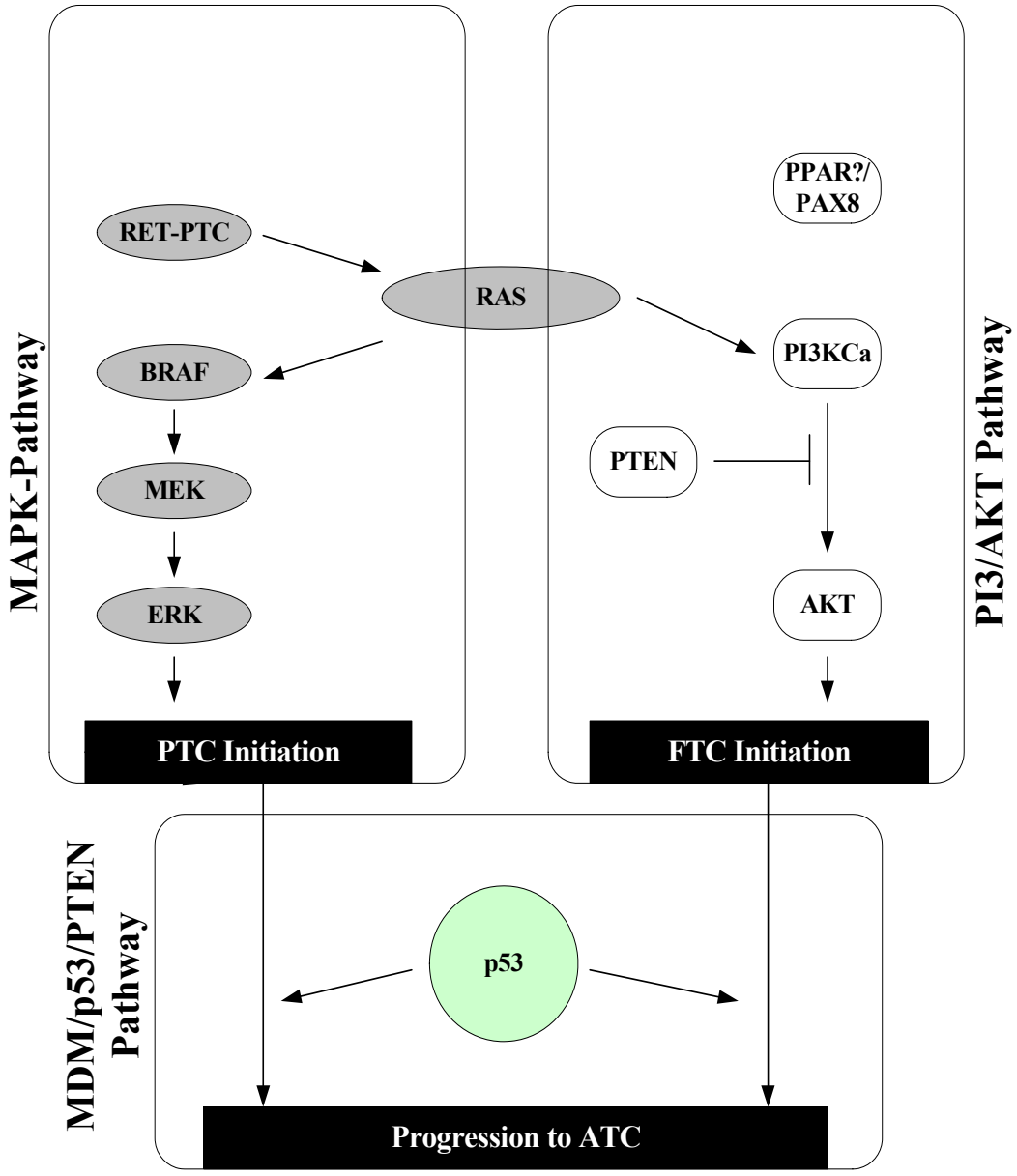

Figure 1-6: The common pathways involved in thyroid pathogenesis. Tumour phenotype may differ in part according to the pre-eminence of the pathway aberrantly activated and its occurrence early or latter in tumour progression (Riesco-Eizaguirre and Santisteban 2007a).

1.2.2.1 Papillary thyroid carcinoma (PTC)

It is the most common type of thyroid cancer, representing more than 85% of all thyroid malignancies (Kumar et al. 2010). It is more common in women and peak in the second and third decade of life. If PTC metastasizes, it does so usually through lymphatogenous route into the regional lymph nodes and rarely through the blood stream. Histologically, the typical lesion presents as an un-encapsulated nodule consisting predominantly from complex branching papillae having fibrovascular cores covered by a single layer of cuboidal epithelial cells, with characteristic nuclear features: a ground glass appearance (large sized optically clear nuclei), nuclear pseudo-inclusion, nuclear grooving with absent or very scanty mitosis. Psammoma bodies may also be seen in approximately half of the cases in the core of the papillae (Rosai 2004). There are over a dozen histological variants of PTC that can mimic other types of thyroid cancer. However, the most common variants are: follicular variant, oxyphilic variant, tall cell variant and diffuse sclerosing variant (Heidinger et al. 1988).

ret rearrangement is the most frequent genetic alteration in PTC. *ret* gene is a proto-oncogene located on chromosomal band 10q11.2, and it encodes a receptor tyrosine kinase (RTK). *ret*-rearrangement leads to the fusion of the *ret*- tyrosine kinase domain with the 5`-terminal regions of heterologous genes, generating chimeric oncogenes designated as *ret*/*ptc* (Santaro et al. 2006). *ret* rearrangement probably represents the early event in thyroid tumour pathogenesis (Tallini et al. 1998).

A high incidence of *braf* gene mutations has been found in PTC ranging from 29% to 83% (Namba et al. 2003). Its prevalence is highest in the tall-cell variant, lower in the conventional variant and lowest in the follicular variant of PTC. But it has been never identified in follicular carcinoma, medullary carcinoma or benign thyroid neoplasms (Xing 2005).

ras mutation plays also an important role in the pathogenesis of PTC, where it is detected in the follicular variant of PTC (10-15% of PTC) (DiCristofaro et al. 2006), and in a subset of PTC that show low rates of lymph node metastasis (Adeniran et al. 2006) and a high tendency to progress (or dedifferentiate) to anaplastic carcinoma (Nikiforov 2004).

It has been shown that *met*-gene activation contributes in PTC carcinogenesis, where it is activated in about 75% of PTC, particularly in the tall cell variant, which has an aggressive clinical course (Nardone et al. 2003).

1.2.2.2 Follicular thyroid carcinoma (FTC)

It is the second most common thyroid cancer, representing approximately 15% of all thyroid malignancies. Clinically, it is presented commonly in middle aged women and elderly individuals. It arises usually *de novo* and rarely from a preexisting benign follicular adenoma. In metastasis, FTC metastases usually through the blood stream into the bone or lung and rarely through the lymphatic route (Evans and Vassilopoulau-Sellin 1998).

Point mutation of *ras*-oncogene is seen in about 45% of FTC (Rivera et al. 2010). The FTC subset harbors this genetic alteration and is associated with poor histological features and poor patient survival (Garcia-Rostan et al. 2003).

pax8/pparγ fusion gene is the second common genetic alteration that is identified in about 26-56% of follicular carcinomas. It results from cytogenetic translocation t(2;3)(q13;25) (Kroll et al. 2000).

PI3K/AKT pathway plays also an essential role in FTC pathogenesis, where activating mutations of proto-oncogene *pi3k* and/or inhibiting mutations of its tumour suppressor gene *pten* are detected in a significant subset of FTC. Coexistence of both abnormalities may contribute to progression and dedifferentiation of FTC to anaplastic carcinoma (Hou et al. 2008).

1.2.2.3 Anaplastic thyroid carcinoma (ATC)

It is a rare thyroid cancer and represents less than 5% of all thyroid cancers. However, it represents the most aggressive form of thyroid cancer with a mortality rate approaching 100% (Kumar et al. 2010). It can develop either *de novo* or from the progression of preexisting well differentiated thyroid carcinoma (papillary or follicular carcinomas). Microscopically, these neoplasms are composed of highly variable morphology cells including: large, pleomorphic giant cells with occasional osteoclast-like multinucleate giant cells; spindle cells with a sarcomatous appearance; or mixed spindle and giant cells. The neoplastic cells express epithelial markers like cytokeratin, but are usually negative for markers of thyroid differentiation, like thyroglobulin.

P53 protein plays a major role in the pathogenesis of this subset of thyroid cancer, where its mutation is detected in up to 40-62% of ATC. P53 is a potent tumor suppressor cellular protein that mediates the arresting of cell cycle in G1 and G2 phases (Dobashi et al. 1994).

PI3K pathway is implicated also in the pathogenesis of ATC, where mutated PI3K proteins have been shown in 12-23% of ATC, just as the downstream effecter of PI3K, AKT protein is activated in up to 93% of the cases (Santarpia et al. 2008).

Aberrations of MAPK signaling pathway also plays an important role in the pathogenesis of ATC, where *ras*- and *braf*-mutations are detected in up to 50% of ATC (Nikiforova et al. 2003, Costa et al. 2008).

1.2.2.4 Medullary thyroid carcinoma (MTC)

It is the only type of thyroid cancer that arises from thyroid C-cells. It may be sporadic in (80%) or familial (20%) in association with familial isolated MTC, multiple endocrine neoplasia syndromes type IIA or IIB (MEN IIA or MEN IIB respectively) (Moley 2003). Sporadic MTC accounts for only 5-9% of thyroid cancer. It presents clinically in the third to fifth decades of life, with a roughly equal proportion of men to women (Doherty 2006).

Histologically, the tumours contain uniform polygonal cells with finely granular eosinophilic cytoplasm and centrally located nuclei with the presence of amyloid, which consists of calcitonin or pro-calcitonin molecules.

Regarding the pathogenesis of medullary carcinoma, germ line and somatic *ret* mutations are responsible for familial and sporadic medullary carcinomas, respectively (Mulligen et al. 1993). *ret* gene encodes the RET-receptors, which are the component of different signalling pathways, including MAPK, PI3K, and JNK that relay the messages to the nucleus to promote cell division (Ichihara et al. 2004).

1.3 Apoptosis in thyroid cancer

Apoptosis is defined as a tightly regulated suicide program in which the cell dies. It involves in different physiological and pathological cellular changes. Apoptosis is involved in normal organs development during embryonic and foetal life and throughout adulthood, where it serves to eliminate unwanted, aged or potentially harmful cells (Kumar et al. 2010). Dysregulation of apoptosis has been implicated also in the pathogenesis of various clinical disorders, including cancer. In fact, aborting apoptosis is regarded as one of the hallmarks of cancer cells (Hanahan and Weinberg 2000).

In the thyroid gland there is increase evidences that apoptosis plays a significant role in the development of thyroid cancer. Potency of thyroid cells to proliferate and/or resist the apoptosis, appear to be different among different types of thyroid cancer. But, in general, it increases with increasing tumour aggressiveness, from well differentiated to poorly differentiated and undifferentiated thyroid cancers.

Among various apoptotic molecules, the FAS/FASL system has been extensively investigated in thyroid cancer. It has been found that thyroid cancer cells express a significant level of FAS, which is negatively associated with the advanced stage of thyroid cancer, and positively associated with the degree of differentiation of cancer. Furthermore, these receptors are functional only when there are certain cytokines and protein inhibitors available (Arscott et al. 1999). However, FAS receptors may be not able to induce apoptosis, and resistance to FAS is found in thyroid cancer cells. The mechanism responsible for this resistance is not known (Mitsiades et al. 1999, Mitsiades et al. 2000).

P53 has also a role in the development of thyroid cancer, where its mutations were found to be important events in thyroid tumour progression once the early stages of oncogene-driven cell transformation had been established (Fagin 2002). In addition, poorly differentiated thyroid cancer cell via the overexpression of p53 can show a significant reduction in cell proliferation and inhibition of malignant behaviour (Moretti et al. 1997). P53 homologue p73 is a member of the p53 protein family and mimics p53 in targeting their gene to promote cell suppression and/or induction of apoptosis. Recently, it was found that this protein has an essential role in thyroid carcinogenesis, where it is dysfunctional in thyroid cancer. Additionally, p73 dysfunction was

suggested to be responsible for unresponsiveness of the neoplastic thyroid cells to DNA- damaging agents, where they fail to elicit cell-cycle arrest and apoptosis in response to chemotherapy (Frasca et al. 2003).

1.4 Role of YAP protein in thyroid cancer

YAP protein expression as well as its role in tumor pathogenesis has been well investigated in different human organs, but not yet in thyroid gland. Therefore, there is neither any idea about the pattern of expression of this protein in normal thyroid tissue and different thyroid lesions, nor about its biological role in thyroid carcinogenesis.

1.5 Working Hypothesis

This study hypothesized that this protein may play an important role in the development, progression or responsiveness of thyroid cancer to chemotherapy through its ability to partner and coactivate a broad spectrum of transcription factors. This hypothesis was analyzed by assessment of 5 different thyroid cancer cell lines (a papillary carcinoma, 2 follicular carcinomas and 2 anaplastic carcinomas) as well as 166 thyroid tissue specimens of different neoplastic and non-neoplastic thyroid lesions in addition to normal thyroid tissues, using RT-PCR, Western blot, Cell proliferation assay, apoptosis assay, cell cytotoxicity assay and immunohistochemistry.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

All listed chemicals were obtained in degree of purity pro analysis.

Chemicals	**Producer**
3-n-morpholino-propansulfonic acid	Merk KgaA (Darmstadt,Germany)
Acetic acid (100%)	J.T. Backer (Deventer, Holand)
Acrylamid-bis-acrylamid (19:1)	MP North America (Solon, USA)
Agarose Ultra Pure™	Invitrogen GmH (California, USA)
Ammonium peroxide sulfate (APS)	Merk KgaA (Darmstadt,Germany)
Bacto™ Agar	Difco Laboratories (New Jersey, USA)
Bacto™ Hefe extract	Difco Laboratories (New Jersey, USA)
Bacto™ Trypton	Difco Laboratories (New Jersey, USA)
Beta-mercaptoethanol	Sigma Aldrich Chemicals (Steinheim, Germany)
Bromphenol blue	Merk KgaA (Darmstadt,Germany)
Carbenicillin	Sigma Aldrich Chemicals (Steinheim, Germany)
Cisplatin	TEVA Pharmaceutical Industries Ltd. (Penssylvania, USA)
Commossie Blue	J.T. Backer (Darmstadt,Germany)
Complete (Proteinase-Inhibitor)	Roche Diagnostic GmbH (Mannheim, Germany)
Developer RP X-OAT EX	Eastman Kodak (Bgndet-Cedex,France)
Dimethylsulfoxide (DMSO)	Sigma Aldrich Chemicals (Steinheim, Germany)
DL-dithiothreitol (DTT)	Sigma Aldrich Chemicals (Steinheim, Germany)
Doxorubicin	Cell Pharm GmbH (Hannover, Germany)
D-PBS (10 x)	Invitrogen GmbH (Carlsbad, USA)
Dulbecco's MEM/ HAM's F-12 (1:1)	Biochrom AG (Berlin,Germany)
Ethanol absolute	J.T. Backer (Deventer, Holand)

Ethidium bromide solution (10 mg/ml)	Sigma Aldrich Chemicals (Steinheim, Germany)
Ethylene diamine tetraacetic acid (EDTA)	SERVA Electrophoresis (Heidelberg, Germany)
Etoposid	Medac GmbH (Wedel, Germany)
Fixer RP X-OMAT LO	Eastman Kodak (Bagndet-Cedex, France)
Foetal calf serum (FCS)	Biochrom AG (Berlin, Germany)
Formaldehyde (37 %)	J.T. Backer (Deventer, Holand)
Formamide	Merk KgaA (Darmstadt, Germany)
G418 sulphate	PPA Laboratories GmbH (Pascing, Austria)
Glycin	Sigma- Aldrich Chemicals (Steinheim, Germany)
Hematoxylin	Dr. K. Hollborne & Söhne (Leipzig, Germany)
Laemmli sample buffer	BIO-RAD Laboratories GmbH (California,USA)
Methanol	J.T. Backer (Deventer, Holand)
Mitoxantron	Baxter (Illinois, USA)
N,N,N',N'- tetramethylethylenediamine	Sigma Aldrich Chemicals (Steinheim, Germany)
Nuclease-free water	Charite Berlin (Berlin, Germany)
Poncceau S	Sigma Aldrich Chemicals (Steinheim, Germany)
RPMI 1640	Biochrom AG (Berlin, Germany)
Skim milk	Difco Laboratories (New Jersey, USA)
Sodium acetate	Merk KgaA (Darmstadt, Germany)
Sodium chloride	Merk KgaA (Darmstadt, Germany)
Sodium citrate	Merk KgaA (Darmstadt, Germany)
Sodium dodecylsulfate (SDS)	Merk KgaA (Darmstadt, Germany)
Sulforhodamin- B (SRB)	Sigma Aldrich Chemicals (Steinheim, Germany)
Tri-chlor acetic acid (TCA)	Merk KgaA (Darmstadt, Germany)
Tris (hydroxymethyl)-aminomethan (Tris-base)	Merk KgaA (Darmstadt, Germany)
Tris (hydroxymethyl)- aminomethane-hydrochloride (Tris-HCl)	Merk KgaA (Darmstadt, Germany)
Triton X-100	Sigma Aldrich Chemicals (Steinheim, Germany)
Trypsin-EDTA-solution 0,5 % / 0,2% (w/v) in PBS (10x)	Biochrom AG (Berlin, Germany)

Tween® 20	SERVA Electrophoresis (Heidelberg, Germany)
Vincristin	harmaceutical Industries Ltd. (Pennsylvania, USA)
Xyline	J.T. Backer (Deventer, Holand)

2.1.2 Biological Materials

2.1.2.1 Human Cell Lines

Cell Line	Tissue of origin and type of lesion	Reference
B-CPAP	Thyroid papillary carcinoma	German Collection of microorganism & Cell Cultures GmbH (Braunschweig, Germany)
FTC-133	Thyroid follicular carcinoma	*Prof Dr. Josef Köhrle
ML-1	Thyroid follicular carcinoma	*Prof Dr. Josef Köhrle
HTH-74	Thyroid anaplastic carcinoma	*Prof Dr. Josef Köhrle
C-643	Thyroid anaplastic carcinoma	*Prof Dr. Josef Köhrle

***** Prof Dr. Josef Köhrle (Charite, Experimental Endocrinology & Endocrinology Research centre of Pathology, Berlin, Germany).

2.1.2.2 Bacteria

Bacteria	Genotype
One Shot®TOP10 (Invitrogen GmbH) (California, USA)	*F mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lac ZΔ M15 ΔlacX74recA1 deoR araD139 Δ(ara-leu)7697 galU galK rpsL(Str^R) endA1nupG*

2.1.2.3 Plasmids

Plasmid	Genotype
pcDNA 3.1/V5-His©TOPO®TA Expression Kit	Invitrogen GmbH (California, USA)

2.1.2.4 Enzymes

Enzyme	Producer
RNase-free DNase Set (50)	Qiagen GmbH (Hilden, Germany)
PstI restriction enzyme	Fermentas GmbH (St. Leon-Rot, Germany)

2.1.2.5 Antibodies

Antibody	Producer
Goat anti- Mouse IgG, HRP- linked	Cayman Chemical Company (Michigan, USA)
Goat anti- Rabbit IgG, HRP- linked	Cell Signaling Technology (Massachusetts, USA)
LATS1 Antibody #9153	Cell Signaling Technology (Massachusetts, USA)
Mouse anti-actin monoclonal antibody	Millipore Corporation (Massachusetts, USA)
Phospho-Akt (Ser473) Antibody #9271	Cell Signaling Technology (Massachusetts, USA)
Phospho-YAP (Ser127) Antibody #4911	Cell Signaling Technology (Massachusetts, USA)
YAP Antibody # 4912	Cell Signaling Technology (Massachusetts, USA)

2.1.2.6 Nucleic acids

Nucleic acid	Producer
Human thyroid total RNA (cat.# 636536)	Clontech (California, USA)
SureSilencing™Plasmid for Human YAP1(Table 2.1)	SABioscience (Maryland, USA)
YAP TaqMan® Gene expression assay (Hs9999- 9901_s1)	Applied Biosystem (California, USA)
18S rRNA TaqMan® Gene expression assay (Hs00- 371735_m1)	Applied Biosystem (California, USA)

Table 2-1: Sequence of the SureSilencing shRNA Plasmid for Human YAP1

Symbol	Reference sequence and Sequence 5' -3'
SureSilencing shRNA Plasmid for Human YAP1 (Cat.No.# KH13459N, SABiosciences)	**Refseq Accession #:** NM_006106 **UniGene #**: Hs.503692 **length:** 21 **Sequence 5' -3'**: shRNA: 1 GGTCAGAGATACTTCTTAAAT 21 mRNA YAP: 941 GGTCAGAGAGATACTTCTTAAAT 961

2.1.3 Kits.

Kit	Producer
20XLumiGlo®Reagent & 20XPeroxide	Cell Signaling Technology (Massachusetts, USA)
Antibody diluent reagent solution	Invitrogen GmbH (California, USA)
Apo Alert® Annexin-V-FITC Apoptosis kit	Clontech Laboratories Inc. (California, USA)
DAKO REAL™ Detection System, peroxidase/ DAB+, Rabbit/ Mouse kit	DAKO (California, USA)
DAKO REAL™Peroxidase-blocking solution	DAKO (California, USA)
DNA Ladder	Fermentas GmbH (St. Leon-Rot, Germany)
DNA Loading dye	Qiagen GmbH (Hilden, Germany)
EndoFree Maxi Kit	Roche Diagnostic GmbH (Mannheim, Germany)
FuGene 6 Transfection Reagent	Roche Diagnostic (Mannheim, Germany)
Mounting medium	R. Langenrinck (Emmendingen, Germany)
Page Ruler™Plus Prestained Protein Lader	Fermentas GmbH (St. Leon-Rot, Germany)
Pierce BCA Protein Assay Kit	Thermo Fisher Scientific Inc. (Massachusetts, USA).
Loading dye	Fermentas GmbH (St. Leon-Rot, Germany)
RNeasy® Mini Kit	Qiagen GmbH (Hilden, Germany)
SuperScript™III Platinum®One-Step Quantitative Kit	Invitrogen GmbH (California, USA)

2.1.4 Equipments

Equipment	Producer
BD FACS Calibur™ Flow Cytometer	BD Bioscience Inc. (California, USA)
Centrifuges:	
. Centrifuge 5417 R	Eppendorf AG (Hamburg, Germany)
. GPK	Beckman Coulter GmbH (Krefeld, Germany)
. GS-6KR	Beckman Coulter GmbH (Krefeld, Germany)
. Optima LE80K Ultracentrifuge	Beckman Coulter GmbH (Krefeld, Germany)
. Table centrifuge 5415 C	Eppendorf AG (Hamburg, Germany)
DNA Thermal Cycler 480	Perkin Elmer, Inc.(Massachusetts, USA)
Electrical power supplier Power Pac 200	Bio-Rad Laboratories, GmbH (California, USA)
Gelelektrophoresis Chamber	Bio-Rad Laboratories (California,USA)
Hypercassette	Amersham Bioscience Europe GmbH (Freiburg, Germany)
Incubator Hera cell 240	Heraeus Instruments GmbH (Osterode am Harz, Germany)
Incubator Kelvitron® t	Heraeus Instruments GmbH (Osterode am Harz, Germany)
Labor scale BL1500S	Sartorius AG (Göttingen, Germany)
LEICA RM 2125 RT Microtome	Leica Microsystem GmbH(Wetzlar,Germany)
Light Microscope	Nikon GmbH (Düsseldorf, Germany)
LightCycler™	Roche Diagnostic (Mannheim, Germany)
Magnetic mixer RCT Basic	IKA Labortechnik (Staufen, Germany)
MP 220 pH-Meter	Mettler Toledo GmbH (Gießen, Germany)
Nalgene Cryos 1 °C Freezing Container	Thermo Fisher Scientific Inc. (Massachusetts, USA).
NUAJRE™ Biological Safety Cabinet	IBS Integra Bioscience (Fernwald, Germany)

Olympus Inverted Microscope IMT-2	Olympus Optical GmbH (Hamburg,Germany)
Oven	Panasonic Electric Works Europe AG (H-olzkirchen, Germany)
Gene Flash Photography of gel-electroph-oresis	Biocompare (California, USA)
Pipetboy acu	Integra Bioscience GmbH (Fernwald, Ger-many)
Pressure cooker	Olympic System Plus (Hamburg, Germany)
PROMAX 1020 Shaker	Heidolph Instruments GmbH & C0. (Schwa-bach, Germany)
Semidry-transfer instrument	Bio-Rad Laboratories (California USA)
Shaker incubator	GFL GmbH (Burgwedel, Germany)
SmartSpec™ Plus Spectrophotometer	Bio-Rad Laboratories (California USA)
Steril-Bank Lamin Air HBB 2448	Heraeus Instruments GmbH (Osterode am Harz, Germany)
Thermomixer 5436	Eppindorf AG (Hamburg, Germany)
Vortex VF 2	IKA- Labortechnik (Staufen, Germany)
Water bath 1083	GFL GmbH (Burgwedel, Germany)
Water purification system Milli-RO 10/ Milli-Q Plus	Millipore Corp. (Massachussetts,USA)

2.1.5 Software

Software	**Producer**
BD CellQuest Pro Software	BD Biosciences (California, USA)
LightCycler Software 3.0	Roche Diagnostic (Mannheim, Germany)
Microplate Manager 5.2.1. (ELISA-Reader)	Bio-Rad Laboratories (California USA)
Microsoft® Office 2007	Microsoft Corporation (Washington, USA)
PASW statistics 18 version 18.0.0	WinWrap® Basic (Alaska, USA)
RealQuant – Relative Quantification Soft-ware 1.0	Roche Diagnostic (Mannheim, Germany)

2.1.6 Disposable materials

Disposable material	Producer
Amersham Hyperfilm™ ECL	GE Health care Limited (Washington, USA)
Cell Culture flasks Petri Dishes and plates	BD Falcon (New Hersy, USA)
Cell scrapper	Sarstedt AG & Co. (Nümbrecht, Germany)
Cover slips	Thermo Scientific (Massachusetts, USA).
Cryotubes Cellstar Cryo.s Greiner	Greiner Bio-One GmbH (Frickenhausen , Germany)
Filter tips Biosphere	Sarstedt AG & Co. (Nümbrecht, Germany)
Gloves Safe Skin Satin Plus	Kimberley-Clark Deutschland (Koblenz-Rheinhafen, Germany)
Light Cycler Capillaries (20µl)	Roche Diagnostic (Mannheim, Germany)
Microscopical slides	R. Langenbrinck (Emmendingen, Germany)
Nitrocellulose Transfer membrane	Schleicher & Schuell BioScience (Dassel, Germany)
Serological pipettes	BD Falcon (New Jersey, USA)
SuperFrost® Plus Objektträger	R. Langenbrinck (Emmendingen, Germany)

2.1.7 Solutions and buffers

1X TBST (pH 7.4-7.6)

Sodium chloride	150 mM
Tris-base	7 mM
Tris-HCl	43mM
Tween 20	0.05 % (v/v)
In dH_2O	

1X Transfer Buffer (pH 8.3) for Western Blot

Glycin	150 mM (w/v)
Methanol	20%
Tris-base	25 mM (w/v)
In dH_2O	

25X MOPS buffer (pH 7.0)

MOPS	5 M
Sodium acetate	12.5 M
EDTA	0.25 M
In dH2O	

50X TAE buffer

EDTA	50mM
Acetic acid	19mM
Tris-base	40mM
In dH_2O	

5X Running Buffer

Glycin	150mM
SDS	0.1% (w/v)
Tris-base	25mM
In dH_2O	

Blocking Solution for Western Blot

Skimmed milk powder	5% (w/v)
In 1X TBST	

LB-Agar

Bacto® Agar	1.5 % (w/v)
in LB-medium	

LB-medium

Bacto®Hefeextract	0.5 % (w/v)
Bacto® Trypton	1.0 % (w/v)
NaCl	0.6% (w/v)
In dH_2O	

P-38 Buffer

0,5M Tris-HCl (pH 6,8)	6 2,5 mM
10% SDS	2%
100% Glycerol	10%
1M DTT in dH2O	50mM
In dH_2O	

S.O.C medium

Bacto®Hefeextract	0.5% (w/v)
Bacto® Trypton	2%
NaCl	10mM
KCl	2.5mM
$MgCl_2$	10mM
$MgSO_4$	10mM
Glucose	10mM
In dH_2O	

Stripping buffer for Western Blot

Glycin	200mM
Tween-20	1%
10% SDS	0.1%
In dH_2O	

SRB- Stain

Sulforhodamin B	0.4% (w/v)
in 1% Acetic acid	

2.2 Methods

2.2.1.1 Cultured conditions, freezing and thawing of cultured human cells

B-CPAP (Human papillary thyroid carcinoma), HTH-74 and C-643 (Human anaplastic carcinoma) were grown in RPMI 1640 medium (Biochrom) supplemented with 10% foetal calf serum (FCS). FTC-133 and ML-1 (human follicular carcinoma) were grown in Dulbecco's MEM/ HAM's F-12 medium (Biochrom) supplemented with 10% FCS. Cell culture was performed with sterile equipments and solutions at a laminar airflow work bench. The cell lines were incubated at 5% CO_2, 95% humidity and 37°C for all cell lines.

Frozen cells were thawed by keeping the cryotubes in a water bath at 37°C for a short period followed by subsequent addition of 15ml pre-warmed medium. Then the cells were centrifuged at 1000 rpm for 5min at room temperature. The supernatant was discarded to remove DMSO (dimethyl sulfoxide), while the cell pellet was resuspended with prewarmed medium, and the resulting cell suspension was transferred to a flask with the appropriate pre-warmed medium and incubated at 37°C. Following incubation for 24h, the medium was replaced with a pre-warmed medium for further incubation.

For freezing, the growing cultured cells were prepared by allowing them to grow up to 80% confluence, at which point they were trypsinized. The obtained cell suspension was diluted with 2x volume of appropriate pre-warmed medium and centrifuged at 1000 rpm for 5min at room temperature. The supernatant was decanted and the cell pellet was re-suspended with pre-warmed medium. After another centrifugation step, 1ml of freezing medium (95% FCS and 5% DMSO) was added to the cell pellet. The total number of cells was determined and the suspension was diluted up to a final recommended cell concentration of each cell line per ml freezing medium. 1 ml of the diluted cell suspension was filled in each cryotube to be subsequently cooled down at a rate 1°C per minute in Nalgene Cryos 1°C Freezing Container , and then at (-80°C) for long-term storage.

2.2.2 Preparation of total RNA from cultured human cells

2.2.2.1 Isolation of total RNA from cultured human cells

As a principle, the total RNA used for quantitative RT-PCR was extracted from growing cultured cell lines using the RNeasy® Mini Kit (Qiagen® GmbH). This kit combines the selective binding properties of a silica-based membrane with the speed of microspin technology. A specialized high-salt buffer system allows up to 100µg of RNA longer than 200 nucleotides to bind to the RNeasy silica membrane.

The cultured cells were trypsinized to be first lysed and then homogenized in the presence of a highly denaturing guanidine-thiocyanate–containing buffer which is 600µl of mixture of RLT buffer and mercaptoethanol 100:1(v/v), which inactivates immediately RNases to ensure purification of the intact RNA. 600µl of 70% ethanol is added to provide appropriate binding conditions, and the sample is then applied to an RNeasy Mini spin column, where the total RNA binds to the membrane and contaminants are efficiently washed away. DNA digestion was performed by incubation with 70µl of RNase-free DNase enzyme (Qiagen® GmbH) for 15min at room temperature. High-quality RNA is then eluted in 30–50µl RNase- free water to be stored at (-80°C) until it is used. With the RNeasy procedure, all RNA molecules longer than 200 nucleotides are purified.

2.2.2.2 Assessment of concentration and purity of the extracted total RNA

The concentration and the purity of total RNA in a mixture can be assessed by UV-light absorbance using a spectrophotometer (Bio-Rad Laboratories GmbH). RNA absorbs UV-light with an absorption peak at 260nm wavelength, whereas proteins (in particular the aromatic amino acids) absorb UV-light at 280nm. By using the Lambert Law, it is possible to relate the amount of the absorbed light to the concentration of the absorbing molecule. An optical density (OD) of 1 corresponds to a concentration of 40µg/ml of RNA.

The purity of the purified total RNA or any nucleic acids with respect to protein contamination was assessed by using the ratio of absorption of UV-light at 260nm versus 280nm. Pure RNA samples have an A260/A280 ratio of 2.0. During this study, only total RNA with a ratio of 1.8-2.1 was used for further investigation.

2.2.2.3 Assessment of integrity the extracted total RNA

The quality of the extracted total RNA was analyzed by electrophoresis in 1% denaturing agarose gel. A denaturing gel system is suggested because most of RNA forms extensive secondary structures via intra-molecular base pairing, and this prevents it from migrating strictly according to its size in gel electrophoresis.

1% agarose gel was prepared by dissolving of 1g agarose (Invitrogen) in 78.2ml distilled water with heating. After complete dissolving, the solution was allowed to cool to 60°C, and then mixed with 17.8ml of 37% formaldehyde and 4ml 25x MOPS.

1µg from each total RNA sample was diluted in 1µl ethidium bromide and 10µl of samples buffer which is a mixture of formamide 50% (v/v), 25x MOPS 4% (v/v), 37% formaldehyde 17.5% (v/v) and dH_2O 28.5% (v/v). The mixtures were denatured at 70°C for 10min. Finally, the denatured samples were loaded into the gel. The electrophoresis separation was carried out at 70V in 1xMOPS running buffer. Afterwards, the gel was analyzed using an UV-transilluminator (Biocompare).

2.2.3 Relative quantification real time RT-PCR

RT-PCR is the most sensitive and reproducible technique currently available for the detection and quantification of mRNA level of genes, because it measures the limited fluorescence at each cycle as the amplification of the target amplicon in the initial template progresses, before limiting reagents including accumulation of inhibitors, or inactivation of the polymerase start to have an effect on the efficiency of amplification. Accordingly, with each amplification cycle, the fluorescence intensity increases proportionally to the increase in amplicon concentration.

The fluorescent reporter molecule used in RT-PCR reaction can be a non-specific DNA dye such as SYBR Green I or sequence-specific probe-based chemistries such as TaqMan, Molecular Beacons and Scorpions (probe, Molecular Beacons and Scorpions) (Heid et al. 1996, Schmittgen et al. 2000).

In addition to the PCR primers, TaqMan probe chemistry includes a third oligonucleotide in the reaction known as the probe. This probe has a fluorescent reporter dye attaching to the 5′ end, and a quencher attaching the 3′end. As long as the reporter and quencher are maintained in close proximity, the fluorescence from the reporter is quenched and no fluorescence is detected. The probe is designed to anneal to one strand of the target sequence just downstream of one of the primers. Since the nuclease activity of *Taq* DNA polymerase runs in the direction from 5′to 3′, so when *Taq* DNA polymerase starts its activity, it encounters the probe, and displaces and degrades the 5′ end of the probe to release the reporter dye into solution that can be detected by the instrument.

The instrument system collects the data for each sample during each cycle, and the resulting plots of fluorescence versus cycle number for all the samples are then set with their background fluorescence at a common starting point (a process known as baseline correction). Then, a threshold level of fluorescence that is set above the background but still within the linear phase of amplification plot, crosses this threshold fluorescence level is called cycle threshold (Ct). The Ct value is directly correlated to the starting target concentration of the sample, where greater amount of the initial DNA-template in the sample is associated with an earlier Ct value.

2.2.3.1 One-step RT-PCR with TaqMan primer

Quantitative real time RT-PCR was performed using SuperScript™ III Platinum® One-Step Quantitative Kit (Invitrogen). The final reaction mixture contained 2µl of purified total RNA of 120ng/µl concentration, 10µl 2x Reaction Mix, 0.4µl of SuperScript™ III RT/Platinum®*Taq*Mix and 1µl TaqMan Primer Probe of gene of interest to a final volume of 20µl per reaction. Reactions were performed in duplicate. RT-PCR was carried out using a Light Cycler Instrument (Roche) using the following program: RT step 50°C for 30min; 1 cycle of 95°C for 10min; 40 cycles of 95°C for 15sec and 60°C for 30sec and finally one step of cooling 40°C for 10sec.

2.2.3.2 Relative quantification

A set of serial standard samples of total RNA were prepared. A gene of interest and housekeeping gene (normalizer), were assessed in these samples to achieve the standard curves for both of them. Using the standard curve, the instrument will automatically displace the efficiency and linearity of the amplification reactions. The optimal standard curve is with a slope in the range of –3.10 to –3.59, which would correlate to a 90–110% efficiency reaction range. Finally the rela-

tive quantification was performed by software using the efficiency-corrected comparative quantisation method. It represents an enhancement of the $2^{-(\Delta\Delta Ct)}$ method used previously. It was introduced in 2001 and it allows the incorporation of different efficiencies for each assay into the mathematical model. First, the relative quantity is calculated separately for each assay, following normalization of gene of interest assays to assigned normalizer. To do this, the following equation was used:

$$\text{Relative quantity to the calibrator} = (1 + E_{GOI})^{\Delta Ct_{GOI}} / (1 + E_{norm})^{\Delta Ct_{norm}}$$

Where:

EGOI = efficiency of the target assay

Enorm = efficiency of the normalizer (Housekeeping gene) assay

$\Delta Ct = (Ct_{calibrator} - Ct_{sample})$

This method eliminates the assumption of equal efficiencies for target gene and normalizer assays (as it is assumed in $2^{-(\Delta\Delta Ct)}$). The data created with light cycler software version 3.5 (Roch), had been used by the Relative Quantification Software "Rel-Quant" to create coefficient files and then to assess the relative quantification in order to express the final result as a (normalized ratio).

2.2.3.3 Statistical test

For assessment of a single gene, the experiment was repeated three times. Each time and for each cell line, a different RNA isolation (i.e., from different passage) were used. The statistical assessment was performed using a student T-test. *P*-values of < 0.05 were considered statistically significant, *P*-values of <0.01 were considered statistically highly significant, and *P*-values of <0.001 were considered statistically very highly significant.

2.2.4 Preparation of total protein from cultured cells

2.2.4.1 Isolation of the total protein from the cultured cells

The cells of each thyroid cancer cell line were plated in 6 well- plates and re-incubated. When the cells reached up to 80% cell confluence in a well, the plates were put on ice and the medium was discarded. After 2 times washing with 1xPBS, the cells were lysed by addition of lyses buffer (Buffer 38) and scraped. This buffer contains SDS, a detergent which lyses the cell compartments to allow the total protein to be extracted. The cell lysates were then transferred to tubes for centrifugation at 14000 rpm for 15min at 4°C. The supernatant was transferred to a new tube and stored at -80°C.

2.2.4.2 Measurement of protein concentration

The concentration of the extracted total proteins was determined by colorimetric quantification using the Pierce BCA Protein Assay kit (Thermo Fisher Scientific Inc.). The assay was done in three steps. The first step is known as biuret reaction, which includes chelation of the Cu^{2+} ion of the protein to Cu^{+1} in an alkaline environment containing sodium potassium tartrate to form a blue chelate complex. In the second step, an intense purple- coloured reaction will result from the chelation of two molecules of bicinochoninic acid (BCA) with the Cu^{+1} that formed in the first step. The BCA/copper complex is water-soluble and exhibits a strong linear absorbance at 470-560nm with increasing protein concentrations.

Following the manufacturer's instructions, a standard curve was established by diluting bovine serum albumin (BSA) solution in 0.9% sodium chloride at defined concentrations. Protein samples were also diluted with 0.9% sodium chloride in 1: 100 and 1:50 ratios. 25µl of each sample of unknown and standard was pipetted into a 96- well microplate. 200µl of the working reagent BCA (50 parts of BCA Reagent A with 1 part of BCA Reagent B) was added to each well and mixed thoroughly on a plate shaker for 30sec. Then the plate was covered and incubated for 30 min at 37°C in the dark. Finally, the plate was allowed to cool and the absorbance was measured at 470nm using ELISA plate reader (BIO-RAD).

2.2.5 Western Blot

Western blot starts with protein separation by electrophoresis on a polyacraylamide gel, followed by transferring the proteins into a nitrocellulose membrane. Then, the membrane was incubated with primary antibody specific to a protein of interest, followed by the membrane's incubation with a secondary antibody that directs to a species-specific protein of the primary antibody. The latter is conjugated with the reporter enzyme horseradish peroxidase (HRP) to be detectable by applying a reagent whose oxidation is catalyzed by HRP. The light that was emitted during the reaction was detected by exposure to an autoradiography film, the light intensity being proportional to the amount of protein. To exclude variations due to the amount of loaded protein or insufficient protein transferring, the membrane should be stripped and re-probed later with β-actin antibody, since it is not influenced by most target proteins and believed to be stable in most cells.

2.2.5.1 SDS-polyacrylamide gel electrophoresis of proteins

First of all, 40µg from each total protein sample was prepared for gel electrophoresis by mixing with Laemmli sample buffer (BIO-RAD) in a ratio of 1:1 (v/v) to be denatured at 95°C for 10 min. Separation of the proteins was conducted by discontinuous electrophoresis in SDS-polyacrylamide gel consisting of 7.5% separation gel (3.75ml Acrylamide-bis- acrylamid (19:1), 150µl 10% APS, 200µl 10% SDS, 7.5ml 1M Tris-HCL (pH 8.8), 15µl TEMED 8,4ml dH_2O), and 4% collecting gel (1ml acrylamide-bis- acrylamide (19:1), 75µl 10% APS, 100µl 10% SDS, 2.5ml 0.5M Tris-HCL (pH 6.8), 15µl TEMED 6,4ml dH_2O). Separation and collecting gel differ from each other by their pH and pore's size. The electrophoresis was started with 60Volt until the protein samples reach the separation gel, afterwards, it was continued at 90 Volt for 1-2 h.

During electrophoresis in a discontinuous gel system, an ion gradient is formed at an early stage of electrophoresis, and it can direct all proteins to be collected into a single sharp band at the border between stacking and separation gel. The gel proteins move then in a constant electric field according to their charge and size. In the gel, polyacrylamide reacts with the SDS to separate proteins according to their molecular weight. SDS binds to proteins and confers a negative charge according to their molecular size to them, ensuring a movement towards the anode of the electrical field.

2.2.5.2 Transfer

In order to make the proteins accessible to antibody detection, they should be transferred from within the gel onto a nitrocellulose membrane using the Semidry-transfer instrument (BIO-RAD). After dissection of the collecting gel, the separation gel was covered by the membrane and together they were sandwiched between two pieces of filter paper to put in the instrument. The blot transfer was set up according to manufacturer's instructions and runs at 3mA/cm^2 for 1 hour. The uniformity and overall effectiveness of protein transfer from the gel into the membrane was subsequently controlled by staining the membrane with Ponceau stain and the gel by Coomassie blue stain.

2.2.5.3 Immunodetection of proteins

The membrane was prepared for detection of specific proteins by incubation for 1h in blocking solution 5% skimmed milk in 0.05 TBST (w/v) at room temperature to prevent any non-specific binding of the antibodies to the membrane. After blocking, the membrane was washed and incubated with the primary and then secondary antibodies according to the following scheme: after washing with TBST, the membrane was incubated with primary antibody overnight at 4◦C. Then washed and re-incubated with the secondary antibody for 1hr at room temperature. After washing with TBST and then TBS, detection of the bounded antibodies was conducted in a dark room using enzyme-linked chemiluminescence detection system 20x LumiGlo® Reagent and 20xPeroxide (Cell Signalling Technology). Following the protocol described in the kit, the ECL reagents A an B were mixed and poured on the membrane for 1min before the membrane was wrapped in plastic film and exposed to XR-film (GE Healthcare).

2.2.5.4 Reprobing of the membrane

The bounded antibodies were stripped off the blot to reprobe the membrane with other antibodies. For this purpose, a stripping buffer (section 2.1.8) was used with vigorous shaking for tow times/1 h for each, followed by washing with strong shaking 5 times/5min for each. The next probing started with the blocking of the membrane.

2.2.6 Stable RNAi-mediated YAP-inhibition in cultured human thyroid cancer cells

RNA interference (RNAi) is a commonplace and popular method for the suppression of the expression of genes of interest in mammalian cells. It was first discovered by introducing a long double-stranded RNA (dsRNA) that was homologous to a specific gene in *Caenorhabditis elegans,* and this resulted in a post-transcriptional silencing of that gene. The dsRNA is initially recognized by an enzyme of the RNase III family of the nucleus, named Dicer, and processed into small dsRNA molecules termed siRNA. siRNA are then bound to the cell RNA-induced silencing complex (SIRSC), a multi-protein complex (with RNase activity), that guides the targeted RNA to degradation (Fire et al 1998).

Downregulation of gene expression mediated by siRNA is transient, and frequently lasts for only 3–5 days in cell culture (Holen et al. 2002). Accordingly, this makes siRNA suitable for many applications, but not for studies of proteins with long half-lives. Another potential problem inherent in transient transfection of siRNA for functional genomics studies is variability in transfection efficiency. This is of particular concern when working with difficult-to-transfect cell lines. In an attempt to overcome these problems, RNAi mediated by a small hairpin RNA or short hairpin RNA (shRNA) has been established (Brummelkamp 2002).

shRNA strategy involves cloning a sequence coding for the sense strand of the siRNA of interest, followed by a spacer and then the equivalent of the anti-sense strand, which ends in a series of 5 U residues. The inclusion of the spacer in the sequence mediates the formation of a hairpin structure, which allowed the sense and antisense sequences to form base pairs. The siRNA expressing cassette (shRNA) annealed to a viral vector. The vector will carry the shRNA and deliver it into a range of cell types for longer-term expression leading to a more persistent silencing effect. Additionally, the vector can pass further on to daughter cells, allowing the gene silencing to be inherited. shRNA hairpin structures are cleaved by the cellular machinery into siRNA, which are then bound to the RNA-induced silencing complex (RISC). This complex binds to and cleaves mRNAs which match the siRNA that is bound to it (McIntyre and Fanning 2006).

2.2.6.1 Preparation of the plasmid-DNA

SureSilencing™ shRNA plasmid for human YAP (SABiosiences) (Figure 2.1 a) was used for stable transfection of the FTC-133 cell line to achieve a stable downregulation of yap-gene expression by shRNA-mediated RNA interference strategy. It encloses shRNA with an insert sequence GGTCAGAGATACTTCTTAAAT. pcDNA 3.1 plasmid (Invitrogen) (Figure 2.1 b) was used as an empty vector to be a negative control for the transfection. Both plasmids are ready for transforming the chemically competent bacteria.

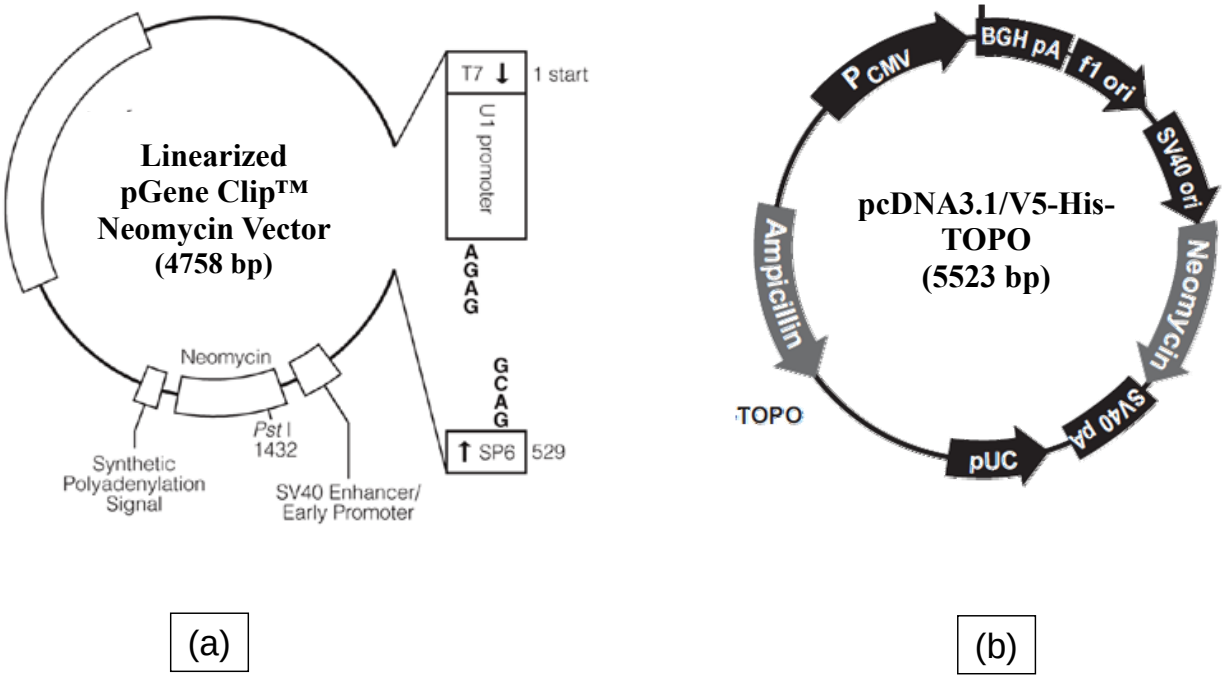

Figure 2-1: Vector maps of the plasmids used to transfect the FTC-133 cell line: (a) SureSilencing™ shRNA plasmid for human YAP (SABiosciences, User Manual Part #1019A Version 1.8); (b) pcDNA3.1/V5-His-TOPO© (Invitrogen, User Manual Catalog no. K4800-40).

2.2.6.2 Transformation of the bacteria with the plasmid-DNA

Following the manufacturer's instructions, each plasmid-DNA is mixed with its chemically competent bacteria One Shot®TOP10 chemically competent *E.coli* (Invitrogen), and incubated in ice for 30min. Then the bacterial suspension with plasmid-DNA was heated briefly in water-bath (42°C) for 30sec and immersed again in ice for 5min. In a bacteriology cabinet, 250µl of S.O.C-buffer was added to the mixture. Then the mixture was incubated for 1h with shaking at 200 rpm at 37°C. After that, 10-50µl of bacterial suspension was inoculated in pre-prepared LB-agar plate and incubated overnight at 37°C, 5% CO_2 to allow the colonization of the transformed bacteria.

2.2.6.3 Screening for recombinant bacterial colonies

To allow only the bacterial clones with successful plasmids transfections, three solitary clones were picked up and each clone was inoculated in a tube containing 2ml of LB-medium supplemented with 20µl carbencillin and 8ml of dH_2O. The new bacterial suspension was re-incubated in a shaker-incubator at 200 rpm at 37°C overnight. This step will allow only the bacterial cells with successful transformation with plasmids to be further cultured and multiplied, while the others with unsuccessful transformation will be killed by carbencillin.

2.2.6.4 Purification of plasmid-DNA

For purification of plasmid–DNA from the transformed bacteria, EndoFree Maxi Kit (Qiagen) was used. For this, 100µl of bacterial suspension was re-incubated in a flask containing 40ml of LB-agar in 160ml sterilized dH_2O supplemented by 400µl of carbencillin in a shaker-incubator overnight with 180 rpm at 37°C. This will allow only the bacterial cells with successful transformation with the plasmid- DNA to proliferate. According to the manufacturer's instructions, the bacterial cells were harvested by centrifugation at 3400 x g (Beckman, Rotor JLA 10.5) for 15min at 4°C. The bacterial pellet was then resuspended completely by vortex with 10ml P1 buffer which already contains RNase. 10ml of P2 buffer was added then and mixed thoroughly by inverting the sealed tube without vortex. After incubating the suspension at room temperature for 5 min, 10ml of P3 was added to the lysate and mixed immediately and thoroughly. Without incubation, the lysate was transferred into the barrel of a QIAfilter Cartridge and incubated at room temperature for 10min. The lysate was then passed through the QIAfilter Cartridge with the aid of a plunger into a 50ml tube. 2.5ml of ER-buffer was added to the filtered lysate and the

mixture was incubated in ice for 30min. During this period, a QIAGEN-tip 500 was equilibrated by 10ml of QBT buffer and the column was allowed to empty by gravity flow. After the clear lysate had passed through the QIAGEN-tip by gravity flow, the tip was washed twice with 30ml of QC buffer. Plasmid-DNA was then eluted with 15ml of buffer QN. The eluted plasmid-DNA was precipitated by the addition of 10.5ml of isopropranolol, mixed and centrifuged for 30min at 4 000 rpm at 4°C. The supernatant was decanted and the DNA-pellet was washed with 5ml of 70% ethanol in endotoxin- free water, and centrifuged further with 15 000 x g for 10min. The DNA pellet was then allowed to air-dry and then re-dissolved in sterilized nuclease- free water.

2.2.6.5 Analysis of Plasmid-DNA by gel-electrophoresis

Plasmid- DNA purity and identity were evaluated using restriction digestion and gel electrophoresis. Enzymatic digestion was performed by mixing 2µg of the purified plasmid-DNA with 1µl of Pst1 restriction enzyme (Fermentas) and 2µl of its buffer and dH_2O up to 20µl in a microcentrifuge tube. For undigested samples, only 2µg of the plasmid-DNA was mixed with dH_2O up to 10µl. After centrifugation for 1min with 2000 rpm, both samples were incubated for 90min at 37ºC in a heating block.

Gel electrophoresis was performed using 1% agarose in 1xTAE buffer (w/v). The whole volume of each of the digested and undigested samples was loaded in the gel to run electrophoresis using 70V for 1-1.5h. Finally, the gel was analyzed using an UV-transilluminator (Biocompare).

2.2.6.6 Transfection of cultured human thyroid cell line with plasmid-DNA

The day before transfection, the growing cultured cells were plated in a 6-well plate, with one well being used for transfection with SureSilencing™ shRNA Plasmid for Human YAP, one well for transfection with the empty vector pcDNA 3.1 plasmid and one well with no transfection as a control. The plate was incubated till the cells reach a confluence of 50-70%. At first, for each plasmid-DNA, a plasmid complex was prepared by mixing 3µl FuGene 6 (Roche) with 97µl FCS-free medium and then the mixture was incubated at room temperature for 5min. After this, 1µg from each plasmid-DNA (SureSilencing™ shRNA Plasmid for Human YAP, pcDNA 3.1 plasmid-DNA) was mixed with its corresponding plasmid complex. The mixtures were vortexed and then incubated for 30min at room temperature. Finally, each mixture was added to a corresponding well and the plate was incubated for further culturing.

2.2.6.7 Antibiotic selection for the introduced plasmid

Both of the introduced plasmids contained a neomycin resistance gene for selection of stably transfected cells (Figure 2-1). Accordingly, after 24-48h, the cells were trypsinized, reseeded in a flask and incubated with their appropriate medium supplemented with neomycin (G418) (PPA Laboratories GmbH) in a final concentration of 400µg/ml. This concentration is the lethal concentration for the FTC-parental cells without transfection after 10 days. The medium was changed every third day until all the cells in the flask of control (i.e., the parental cells without any transfection) were dead. At that time, all the remaining cells in the other two flasks with transfection were considered to be resistant to neomycin because of the successful transfections.

2.2.6.8 Cloning of the stably transfected cells

Once the non- transfected cells had died off, neomycin- resistant colonies were trypsinized and reseeded in a 96 wells-plate to achieve 1cell/well. This is in order to establish stably transfected monoclones for each plasmid-DNA. After further culturing, each transfection resulted in several transfected monoclones for each plasmid.

2.2.6.9 Assessment of the extent of the knocking-down of yap-gene in the transfected clones

Total RNA from the transfected monoclones was isolated, and their concentration, purity and integrity were assessed as described in section 2.2.2. 2µl of total RNA with a concentration of 120 ng/µl from each sample was assayed using one-step TaqMan real time relative quantification RT-PCR as described in section 2.2.3. To confirm the expression of YAP mRNA, total protein was isolated from the cells of the transfected clones and assessed by Western blot for YAP-protein expression as described in sections 2.2.4 and 2.2.5.

2.2.7 Cell proliferation assay

Cells were plated in 96-well plates as 300cells/well and incubated with their cultured medium for 5 successive days. Each day, the rate of cell proliferation for each clone was assessed by Sulforhodamine- B (SRB) assay method. The assay was started by pouring out the medium gently and then fixation of the cells by adding 200µl of cold (4ºC) 10% trichloroacetic acid (TCA) followed by incubation of the plate at 4ºC for 1h. After 5 times washing with tap water to remove the excess TCA, medium and low molecular weight metabolites and serum proteins, the fixed cells were stained with 100µl of 0.4% of SRB solution for 10min. Then excess SRB was decanted, and the plates were quickly rinsed 5 times with 1% acetic acid, and then left at room temperature for drying overnight. Next day, 300µl of 20M tris-base was added and after 20-30min with shaking, the plates become ready for measurement of absorbance at 562nm by ELISA-reader (BIO-RAD) (Skehan et al. 1990).

2.2.8 Cell apoptosis assay using annexin-V and propidium iodide

Apoptosis is characterized by variable morphological features. However, the changes in the plasma membrane are of the earliest features. In apoptotic cells, the membrane phospholipids phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment (Martin et al. 1995). Because externalization of PS occurs in the earlier stages of apoptosis, annexin-V staining can identify apoptosis at an earlier stage than assays based on nuclear changes such as DNA fragmentation. Annexin-V staining precedes the loss of membrane integrity which accompanies the latest stages of cell death resulting from either apoptotic or necrotic processes. Therefore, staining with annexin-V in conjunction with vital dyes such as propidium iodide (PI) allows the identification of early apoptotic cells (annexin-V positive and PI negative).

The assay performed by platting the cells into six well-plates as 2 x10^5cell/well, and incubated for 24 h. Next day, sets of cells were treated with three different concentrations of cisplatin (0mM, 10mM and 20mM) and another sets of cells were treated with doxorubicin (0µM, 0.2µM and 0.5µM) for 48h. Apoptosis assay was performed using ApoAlert® Annexin V-FITC Apoptosis Kit (Clontech) according to the manufacturer's instructions. In each well, the medium was

collected, the cells were washed with 1xTBS followed by trypsinization. The medium, washing's TBS and the trypsinized cell suspension were collected together in a tube to be centrifuged with 1000rpm, for 5min at room temperature. The supernatant was decanted and the cell pellet re-washed with 1xTBS and then with binding buffer followed each time by centrifugation at 1000rpm, for 5min at room temperature. Finally, the cell pellet was resuspended with 215µl of staining mixture (200µl binding buffer, 10µl propidium iodide and 5µl Annexin-V). After gentle vortex, the cells suspension was incubated in the dark at room temperature for 15min. Then and just before the assay, 285µl binding buffer was added to each sample to assay by BD FACS Calibur™ Flow Cytometer (BD. Bioscience).

2.2.9 Cell cytotoxicity (viability) assay

The cells were plated in 96 well- plates with equal number, and incubated for 48h to allow their adherence to the bottoms of the wells and for further growing. After this time, the cells were treated with a series of different concentrations of a cytotoxic drug and reincubated for 4 successive days. The rate of cell proliferation (i.e., the survived cells against cytotoxic drugs), was assessed by SRB-Assay as described in section 2.2.7.

2.2.10 Immunohistochemistry (IHC)

2.2.10.1 Patients and tissue samples

The study included 166 thyroid lesions. The lesions were classified according to their histopathological diagnosis into 4 major groups: normal thyroid tissue; non-neoplastic; benign neoplastic and malignant neoplastic lesions (Table 2-2). Both thyroiditis and hyperplasia cases were included in the non-neoplastic lesions, while the neoplastic lesions were classified according to the diagnostic criteria of World Health Organization (WHO) histological classification of thyroid tumours (Hedinger et al. 1988).

Table 2-2: The Number (%) of the included cases in relation to the histopathological diagnosis, sex, and age.

type of lesions	Diagnosis	Total No. (%)	Sex		Mean of age ± SD	
			Male N0. (%)	Female No. (%)	Male	Female
Normal	Normal	10 (6.02%)	5 (50%)	5 (50%)	53.4	46.2
Non-neoplastic Lesions	Thyroiditis	17(10.24%)	2 (11.7%)	15(88.24)	64.5	43.13
	Hyperplasia	25(15.06%)	5 (20%)	20(80%)	56.8	54.7
Benign Neoplastic Lesions	Follicular Adenoma	25(15.06%)	9(36%)	16 (64%)	55.5	50.625
Malignant Neoplastic Lesions	Papillary Carcinoma	22(13.25%)	10(45.4%)	12(54.55%)	56.9	53.83
	Follicular Variant-Pap. Ca.	23(13.86%)	7(30.4%)	16 (69.57%)	62.1	50.93
	Follicular Carcinoma	18(10.84%)	7(38.8%)	11(61.12%)	58.7	58.9
	Anaplastic Carcinoma	9(5.42%)	5(55.5%)	4(44.45%)	70	69
	Medullary Carcinoma	17(10.24%)	9(52.9%)	8(47.06%)	58	63.12
Total		166(100%)	59	107		

2.2.10.2 Immunohistochemical staining of YAP & Phospho-YAP (Ser-127)

The principle of the IHC procedure is to visualize a target antigen by enzymatic reaction after creating an interaction with the specific antibody. The IHC protocol consists of three key steps: blocking, epitope recovery and detection.

Blocking prevents the background staining resulting from any non-specific binding of the antibody to endogenous peroxidase, alkaline phosphatase, avidin and biotin. Epitope recovery step is often required for formalin fixed paraffin-embedded samples to unmask the epitopes that are altered during fixation and processing. For this purpose, a number of heat-based treatment techniques have been developed including water baths, microwaves, autoclaves, and pressure cookers. The heat can unmask epitopes by disrupting cross-links formed by formalin fixation and by changing the tertiary protein structure. Among these heating methods, water bath system has shown fairly good results for a wide range of antibodies and tissues. For certain antibodies in which heat is not recommended, enzymatic treatment using trypsin or pepsin may be an alternative. Next, the target antigen is detected by direct enzyme-based techniques such as horseradish peroxidase (HRP) or alkaline phosphatase (AP). The antigen-antibody complex is finally visualized by the addition of a chromogen such as 3,3`-diaminobenzidine (DAB) or 3- amino-9-ethylcarbazole (AEC), which stains brown or red respectively. The choice of chromagen depends on colour preference and solubility. Where not specified, steps are performed at room temperature. Positive and negative controls should be used in each assay to ensure quality staining (Hayat 2006).

Immuohistochemistry was performed by deparaffinization of 4μm thickness tissue sections in xylene and then dehydration within a graded alcohol series. Endogenous peroxidase was blocked by treatment of the sections with hydrogen peroxidase blocking solution (Dako) for 10min. By pressure cooking of the tissue sections, the antigen was retrieved with 1% sodium citrate buffer (pH 6.0) for 5min. The sections then were incubated for 1h at room temperature with primary antibody at the recommended dilution (1:100 for anti-YAP and 1:50 for anti-Phospho-YAP Ser-127). Immunohistochemistry analysis was conducted using a biotin-streptavidin immunoperoxidase method with diaminobenzidin in 0.03% hydrogen peroxidase (DAKO REAL™ Detection System, Peroxidase/DAB+, Rabbit/Mouse). The positive tissue control for YAP included colonic carcinoma tissue. The negative control included a section incubated with the primary

antibody diluent without primary antibody. As well as in each stained tissue section, the smooth muscles of blood vessels and leukocytes were used as the internal positive control, and internal negative control respectively.

2.2.10.3 Scoring of immunohistochemical staining

The immunohistochemical staining pattern of the nuclei and the cytoplasm of the lesion cells in each section was assessed by other two pathologists (RA and MK) using Remmele Scoring system (Remmele 1987). This system is based on both the percentage of positive cells and the staining intensity. The percentage of positive cells was estimated on a four-tier scale 1 (<10%), 2 (≥11-50%), 3 (≥51-80%), 4 (≥80%). A four-tier scale was also used to score the staining intensity 0 (negative), 1 (weak), 2 (moderate), 3 (strong). The staining intensity was based on the predominant staining intensity in the lesion. The final staining immunoreactivity (IR) score was obtained by multiplying the two scores. A final score of 0 was classified as negative, 1-3 as low, 4 -6 as moderate, and 8-12 as high.

2.2.10.4 Statistical test

The data of immunohistochemical assessment was tested statistically using PASW statistics 18 program. The variables were analysed by using the Mann-Whitney-Test. The difference is statistically significant when $P < 0.05$, highly significant when $P\text{-}value < 0.01$, and very highly significant when $P\text{-}value < 0.001$.

3 Results

3.1 Expression and phosphorylation of YAP-protein in thyroid cancer cell lines

3.1.1 Expression of total YAP mRNA in thyroid cancer cell lines

3.1.1.1 Assessment of total RNA integrity by gel-electrophoresis

After measuring the concentration and assessment of its purity, the integrity of the purified total RNA from different cell lines was assessed by RNA-gel electrophoresis as described in section 2.2.2.3 (Figure 3-1).

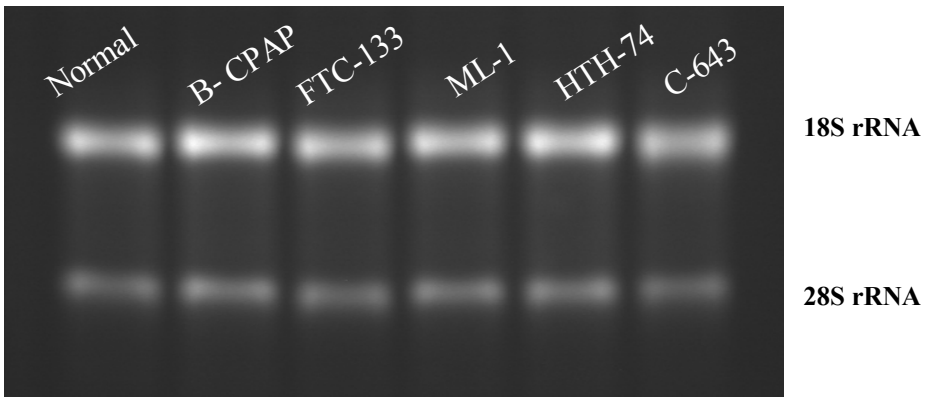

Figure 3-1: Gel-electrophoresis of the total RNA of different thyroid cell lines.

It shows the 2 bands of 18S rRNA and 28S rRNA are in the ratio of 2/1. This indicates that the isolated total RNAs were fully integrated and suitable for RT-PCR.

3.1.1.2 Expression of YAP mRNA in different thyroid cancer cell lines by RT-PCR

A commercially available total RNA from normal thyroid tissues (Clontech) was used as a calibrator. Relative quantification of YAP mRNA was performed using "Rel-Quant" software and 18S rRNA as a normalizer (Housekeeping gene) as described in section 2.2.3 (Figure 3-2).

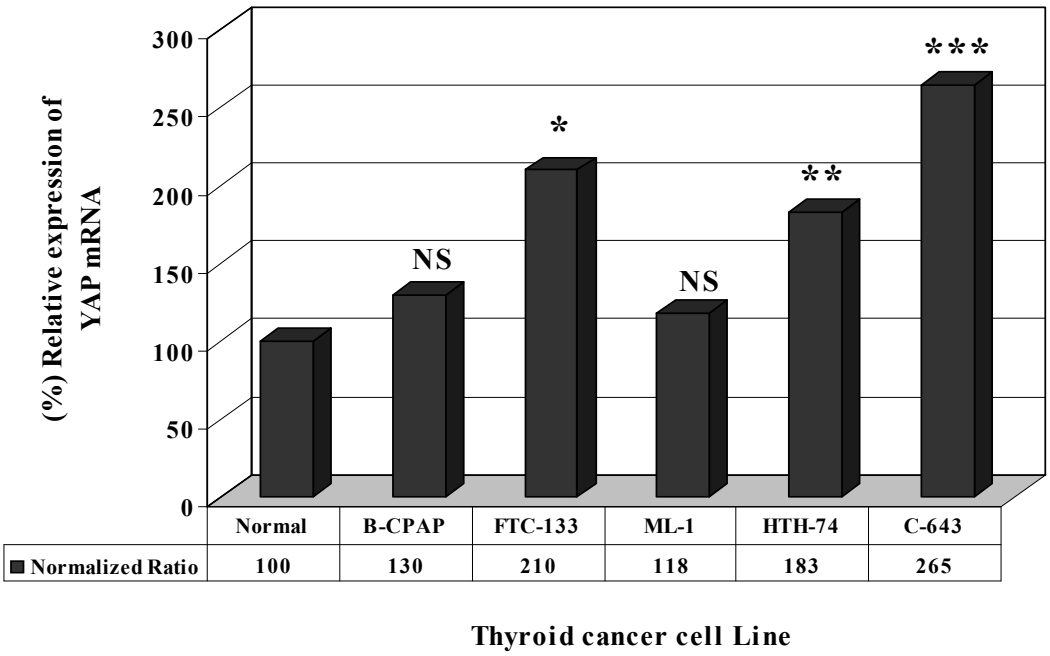

Figure 3-2: The % of normalized ratio of different thyroid cancer cell lines in comparison to the normal thyroid tissue (NS: not significant *P-value*> 0.05, *: significant difference *P-value* < 0.05, **: highly significant difference *P-value* < 0.01, *: very highly significant difference *P-value* < 0.001).**

It shows that all thyroid cancer cell lines show an increased YAP mRNA expression in comparison to the normal. However, this increment is variable where it is duplicated in C-643 and FTC-133 (265 and 210, respectively), but it is much smaller in the others. Statistically, the increment in the expression of YAP mRNA is significant in FTC-133 (*P-value*< 0.05), highly significant in HTH-74 (*P-value*< 0.01), very highly significant in C-643 (*P-value*< 0.001). But in other cell lines, ML-1 and B-CPAP, the increment is not significant (*P-value*> 0.05).

3.1.2 Expression of YAP-protein in thyroid cancer cell lines by western blot

From each thyroid cancer cell line, 40µg of total protein was loaded in the SDS- polyacrylamid gel. Anti-YAP antibody (#4912, Cell Signalling Technology) was used in a dilution of 1:1000 and overnight incubation at 4 ºC to detect the total endogenous YAP protein (Figure 3-3a). Stripping and reprobing of the membrane with β-Actin antibody (Millipore) was used as a control with a dilution of 1:5000 and incubation overnight at 4 ºC (Figure 3-3 e).

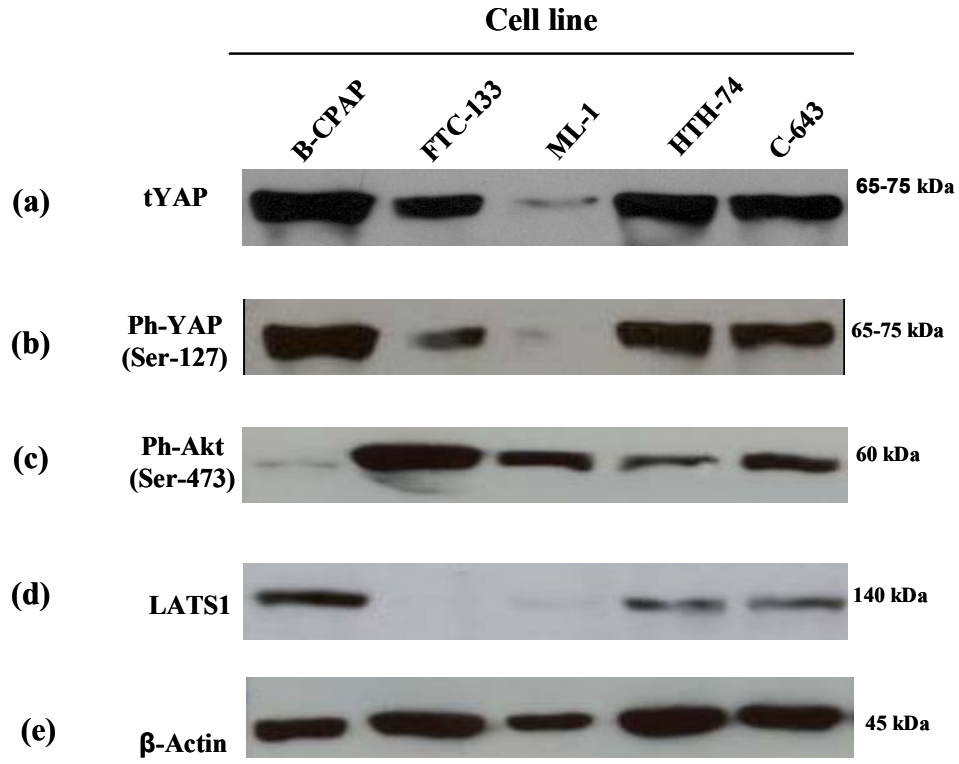

Figure 3-3: Western blot of total protein from different thyroid cancer cell lines to detect the expression of YAP-protein in different cancer cell lines (a), the proportion of phosphorylated YAP Ser-127 (b), and its coexpression with Phospho-AKT (Ser-473) (c) and LATS1 (d). (e) β-actin as a housekeeping protein.

It shows a high expression of YAP-protein in all thyroid cancer cell lines except ML-1 and this result is consistent with the result of YAP mRNA expression in (figure 3-2).

3.1.3 Phosphorylation of YAP- protein in thyroid cancer cell lines

40µg from the same samples was assessed by western blot using anti-Ph-YAP Ser-127 (#4911, Cell Signaling Technology) in a dilution of 1:1000 overnight at 4ºC. This antibody can detect specifically only the YAP-protein that is phosphorylated at Ser-127 (Figure 3-3 b). Stripping and reprobing the membrane for β-Actin was performed as a control for technical problems of loading.

It shows that the bands of Ph-YAP Ser-127 are nearly identical to those of the total YAP-protein (figure 3-3 a) regarding their thickness and intensity. This suggests that most of the endogenous YAP-protein in these cell lines, actually is phosphorylated at Ser-127.

3.1.4 Co-expression of Phospho-AKT and LATS1 in thyroid cancer cell lines

40µg from the same samples that was used for YAP and Ph-YAP Ser-127 was assessed in the same way for the expression of Ph-AKT Ser-473 and LATS1 (Cell Signalling Technology) with a dilution of 1:1000 and incubation overnight at 4ºC for each (Figure 3-3 c and d, respectively). The same membrane was stripped and reprobed for β-Actin as a control.

They show that both of the cell lines of anaplastic carcinoma (HTH-74 and C-643) show a strong co-expression of both Ph-AKT Ser427 and LATS1. Meanwhile, the papillary thyroid cancer cell line (B-CPAP) shows a strong co-expression of LATS1 but undetectable Ph-AKT Ser- 473. In contrast, the follicular cell lines FTC-133 and ML-1 show a strong co-expression of Ph-AKT Ser-127 but undetectable LATS1.

3.2 Assessment of the successfulness of stable transfection of FTC-133 cell line with DNA-plasmids

A follicular cancer cell line, FTC-133, was transfected with SureSilencing™ shRNA Plasmid for Human YAP (SABiosiences), to knock down YAP expression. pcDNA3.1 Plasmid (Invitrogen) is an empty vector and it was used to transfect the same cell line to be used as the negative control of the transfection. Cell transfection with the DNA-plasmids was performed as described in section 2.2.6.6. Total RNA from the parental cells and from the cells of the transfected monoclones was isolated and then their concentration, purity and integrity were assessed as described in sections 2.2.2. YAP mRNA expression was assessed by One-Step relative quantification RT-PCR using 18S rRNA as a normalizer (house keeping gene) and the parental cells of FTC-133 as the calibrator for the relative quantification using (RelQuant Soft ware) as described in section 2.2.3 (Figure 3-4).

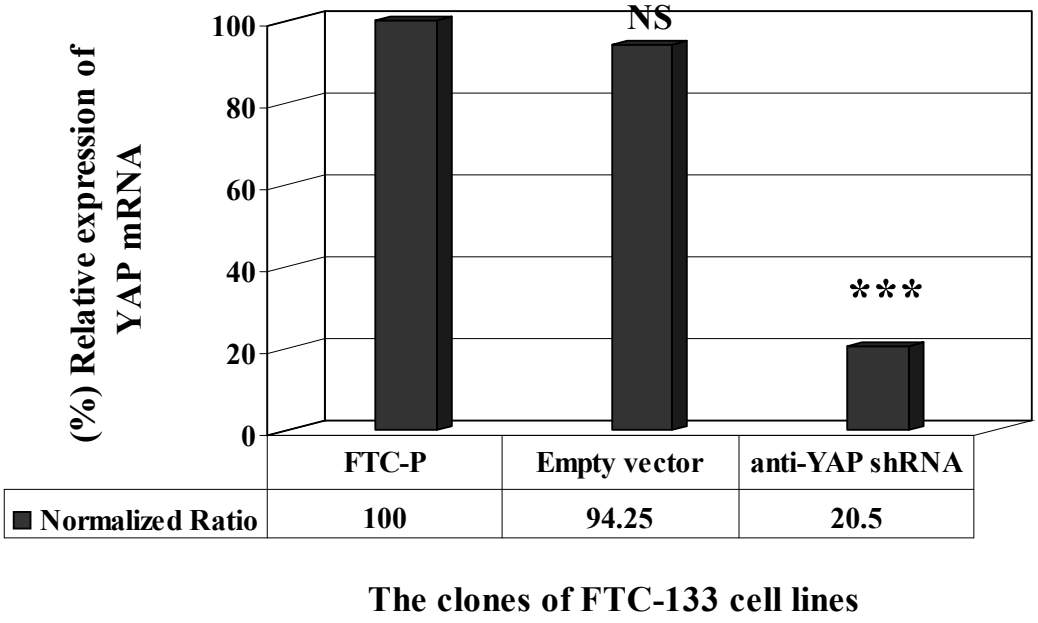

Figure 3-4: Relative quantification of gene expression of YAP in transfected clones of FTC-133. YAP mRNA expression in transfected clones in comparison to the parental cells (NS: no significant difference *P-value* >0.05, *: *very highly significant difference P-value*<0.001).**

It shows that YAP mRNA expression in the monoclone with anti-YAP shRNA is knocked down to 20.5% of that in the parental cells, while in the monoclone of the empty vector, its expression is nearly identical to that of the parental cells (94.25%).

YAP mRNA expression in these clones was confirmed by immunoblotting of their total protein extracts with anti-YAP antibody (#4912, Cell Signalling Technology) as described in section 2.2.5 (Figure 3-5).

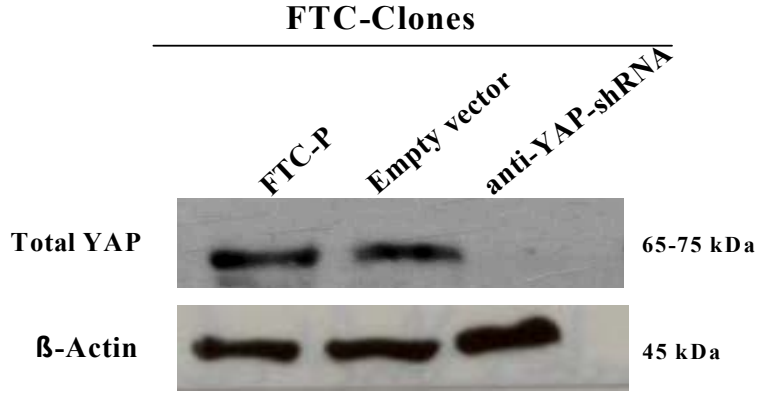

Figure 3-5: YAP-protein expression in Western blotting of the total protein extract from the transfected monoclones in comparison to the parental cells.

It confirmed YAP mRNA expression in RT-PCR, where the blot with anti YAP antibody shows nearly identical bands in the lanes of the parental cells and the monoclone of the empty vector, while the lane of anti-YAP shRNA monoclone shows an undetectable band for YAP-protein.

3.3 Relation of YAP-protein to the rate of proliferation of thyroid cancer cell line (FTC-133).

In these cells, the doubling time was assessed by SRB-assay as described in section 2.2.7. The absorbance was measured for the cells each day for 5 successive days. For each clone, the absorbance on the first day was considered as the basal value, and on each successive day, the absorbance of the accumulated cells was calculated as the percentage (%) based on the absorbance on the first day (Figure 3-6).

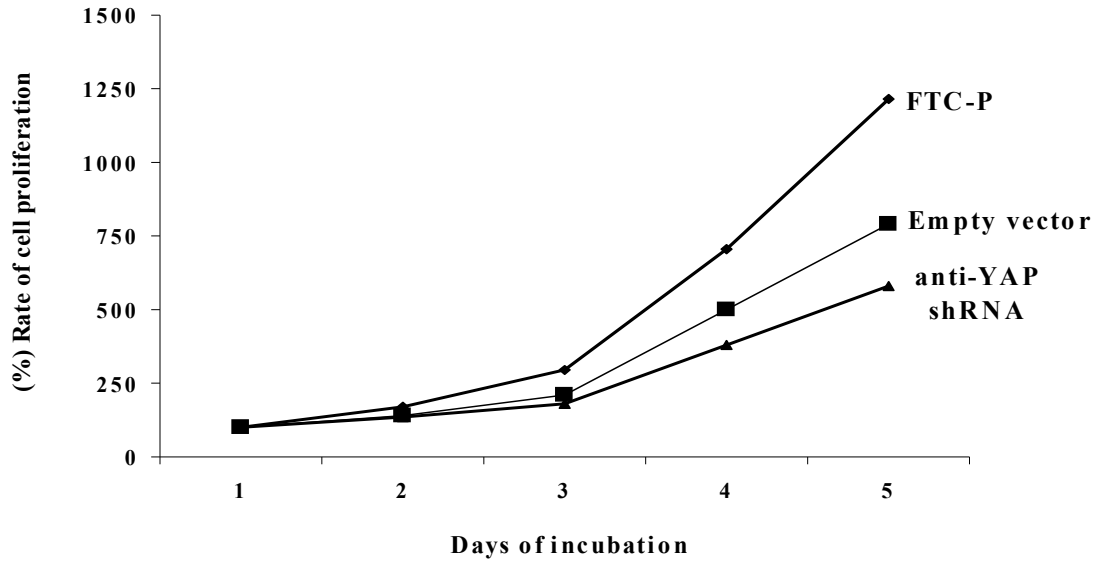

Figure 3-6: Correlation of the cell proliferation with the level of YAP-protein expression in the thyroid cancer cell line FTC-133

It shows that the doubling time in the cells of monoclone with anti-YAP shRNA is almost always lower than that in the parental cells and the monoclone of empty vector. Additionally, the difference increases with the duration of incubation.

To show whether this difference has any significant statistical significance, the doubling time on each day in the transfected clones is compared to that of the parental cells (Figure 3-7).

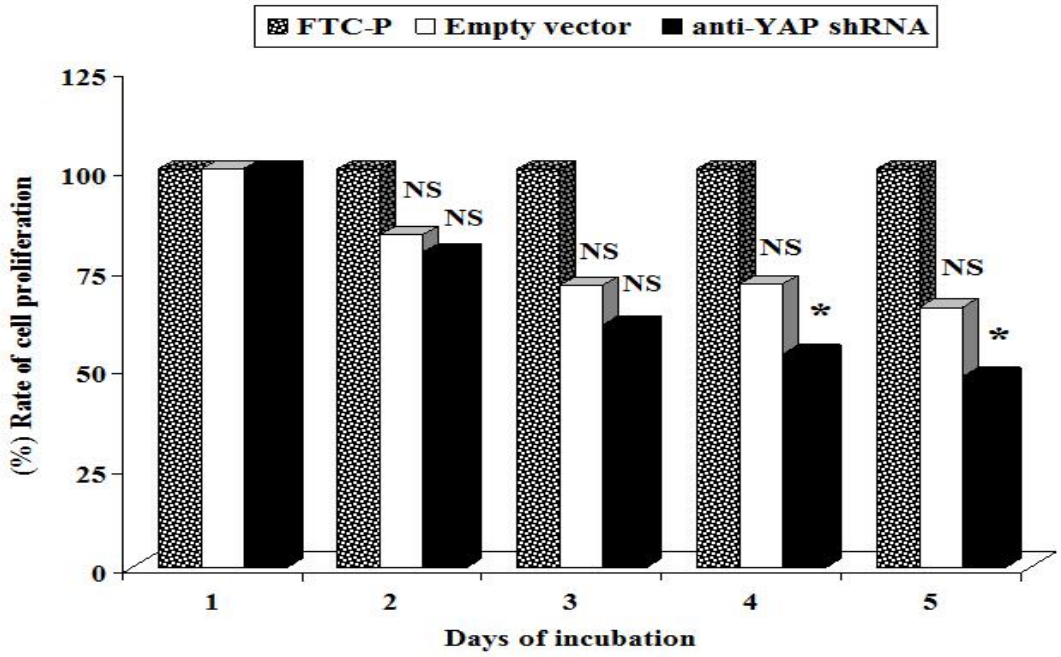

Figure 3-7: The rate of cell proliferation in each clone in comparison to that of the parental cells on each day for 5 successive days (NS: not significant *P-value*> 0.05, *: significant difference *P-value*< 0.05).

In comparison to the parental cells, the rate of doubling the monoclone with anti-YAP shRNA shows an obvious reduction on the last two days. Statistically, this difference is significant (*P-value* < 0.05). At the same time, the rate of cell doubling in monclone with empty vector shows also a lower rate of cell proliferation in comparison to that of parental cells. However, this difference has no statistical significance (*P-value* > 0.05).

3.4 Relation of YAP-protein with the apoptosis of thyroid cancer cell line (FTC-133) in response to chemotherapy

3.4.1 Relation of YAP-protein with the apoptosis of thyroid cancer cell line (FTC-133) in response to cisplatin

The rate of apoptotic cells in the parental and in the transfected cells was assayed as described in section 2.2.8. The apoptosis was induced by incubating the cells for 48 hours with cisplatin in three different concentrations (0mM, 10 mM and 20 mM). The rate of apoptotic cells in the transfected cells was compared with that of the parental (FTC-133) cells (Figure 3-8).

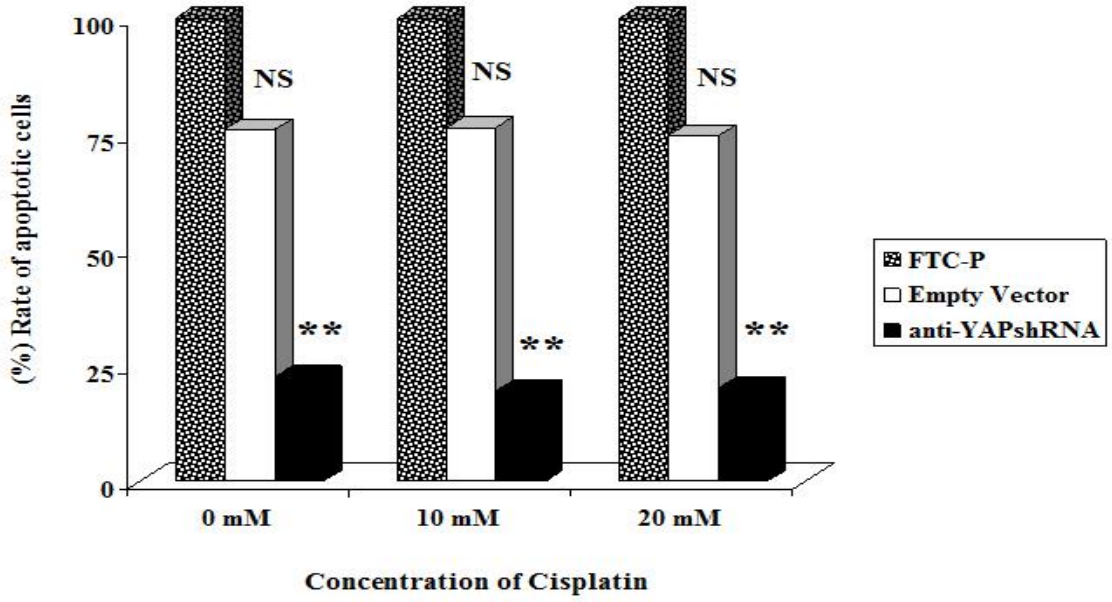

Figure 3-8: The rate of apoptosis in response to cisplatin in the transfected cells in comparison to the parental cells (NS= not significant *P-value* < 0.05, **= highly significant difference *P-value* < 0.01).

Statistically, the figure shows a highly significant reduction in the rate of apoptosis in the cells of monoclone with knocking down of YAP-protein in comparison to the parental cells in all the three concentrations (*P-value*< 0.001). At the same time, the rate of apoptosis in the cells of the monoclone of empty vector shows no significant difference from that of the parental cells (*P-value* > 0.05).

3.4.2 Relation of YAP-protein with the rate of apoptosis of thyroid cancer cell line (FTC-133) in response to doxorubicin

The rate of apoptosis in the parental (FTC-133) cells and in the transfected cells in response to doxorubicin was assayed in the same way as for the cisplatin. The cells were treated with three different concentrations of doxorubicin (0nM, 0.2nM, 0.5nM) (Figure 3-9).

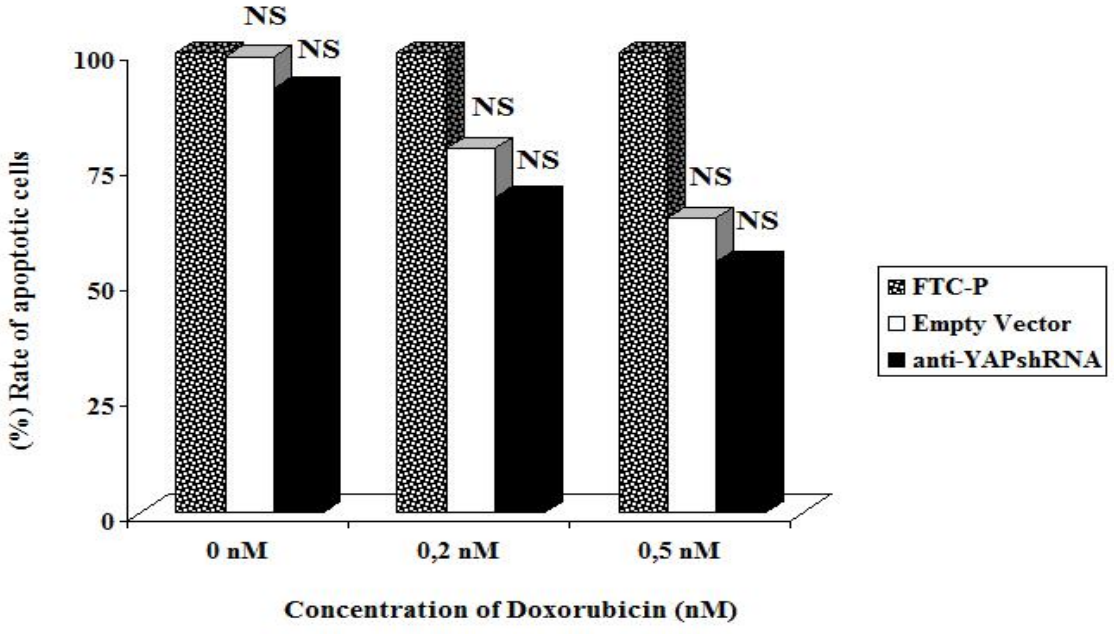

Figure 3-9: The rate of apoptosis in response to doxorubicin in the transfected cells in comparison to the parental cells (NS= *P-value*< 0.05).

The figure shows that neither the cells of monoclone with anti-YAP shRNA nor those of empty vector monoclone show a significant reduction in the rate of apoptosis. Additionally, only a slight difference can be seen in the rate of apoptosis between the cells of anti-shYAP and those of monoclone of empty vector. Statistically, and in comparison to the parental cells, the rate of apoptosis in the transfected cells shows no significant difference (*P*-value> 0.05).

3.5 Relation of YAP-protein with the resistance of thyroid cancer cell line (FTC-133) to chemotherapy

3.5.1 Relation of YAP-protein with the resistance of thyroid cancer cell line (FTC-133) to chemotherapy with cisplatin

The cell cytotoxicity (surviving) with cisplatin was assessed by measuring the rate of survived cells by SRB-assay after incubating them with a serial concentration of cisplatin as described in section 2.2.9. In each concentration gradient of cisplatin, the rate of the survived cells in the transfected cells was compared to that in the parental FTC-133 cells (Figure 3-10 a).

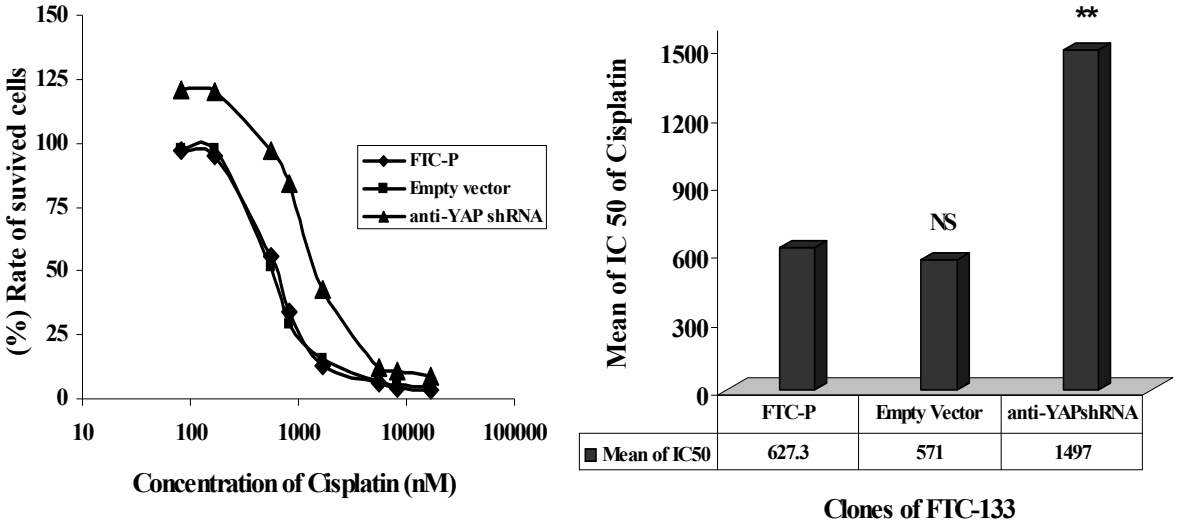

Figure 3-10: (a) The rate of the survived cells in cisplatin in relation to the level of YAP-protein expression. (b) Correlation of the rate (%) of IC50 with cisplatin in the transfected cells to that in the parental FTC-133 cells (NS: *P-value* > 0.05, **: highly significant difference *P-value* < 0.01).

Figure 3-10a shows that in comparison to the curve of the parental cells, the curve of anti-YAP shRNA monoclone shifts to the right, while the curve of the monoclone with empty vector runs nearly identical to that of the parental cells. This means knocking down of YAP-protein increases the resistance of the cancer cells to the cisplatin that in turn presented as an increase in its survival rate when incubated with cisplatin.

To confirm this finding, the IC50 to cisplatin in these cells was calculated. Then, the IC50 in the transfected monoclones was compared with that of the parental FTC-133 cells (Figure 3-10 b). It shows that the IC50 of the cells after knocking down of YAP-protein is markedly increased in comparison to the parental cells, and this increment statistically is highly significant (*P-value*< 0.001). This finding confirmed that the resistance of FTC-133 thyroid cancer cells to cisplatin is markedly increased after knocking down of YAP-protein.

3.5.2 Relation of YAP-protein with the resistance of thyroid cancer cell line (FTC-133) to other cytotoxic drugs.

In the same way as with cisplatin, the same cells were treated with a serial concentration of other four cytotoxic drugs: doxorubicin, mitoxantron, vincristin and etoposide (Figure 3-11).

It could be seen that the curve of the cell survival rate for monoclone of anti-YAP shRNA and empty vector shifts in nearly identical way to each other and to that of the parental cells with different cytotoxic drugs. This means that knocking-down of YAP-protein has no effect on the rate of survival of these cells with these cytotoxic drugs: doxorubicin (a), mitoxantron (c), vincristin (e), etoposide (g).

To confirm these findings, the IC50 of these cells with each cytotoxic drug was calculated. Then, the IC50 of the transfected cells was compared with that of the parental FTC-133 cells: doxorubicin (b), mitoxantron (d), vincristin (f), etoposide (h).

It can be shown that there is no obvious difference in the IC50 of the cells with or without knocking-down of YAP-protein with the four cytotoxic drugs. Statistically, all these differences are not significant where *P-value* > 0.05.

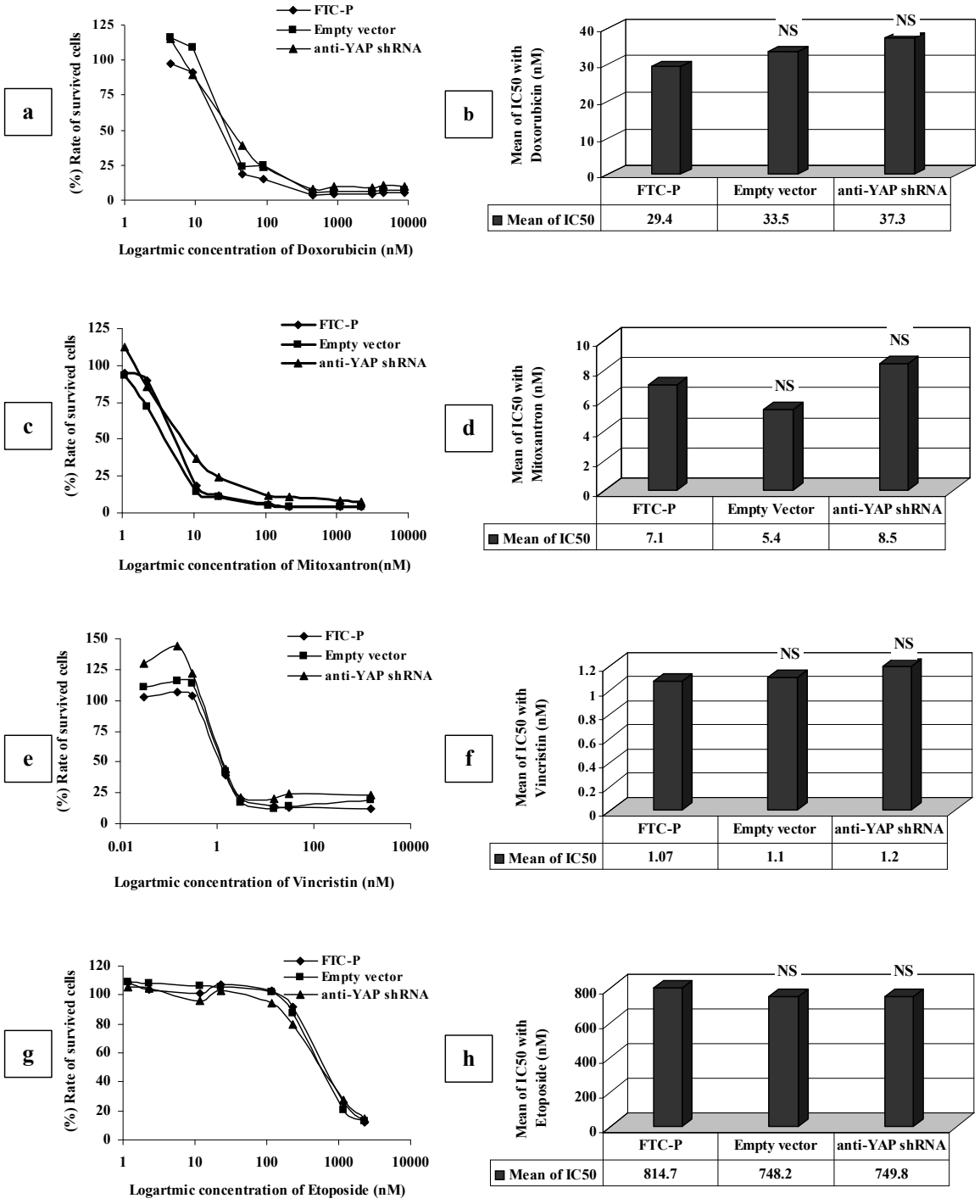

Figure 3-11: The rate of the survived cells and IC50 of the transfected clones in relation to the parental cells of FTC-133 cell line with cytotoxic drugs: Doxorubicin (a, b); Mitoxantron (c, d); Vincristin (e, f); Etoposide (g, h) (NS: not significant difference *P-value* > 0.05).

3.6 Immunohistochemistry

3.6.1 Immunohistochemical expression of total YAP-protein in different thyroid lesions

A representative histological section from each thyroid lesion was stained immunohistochemically with anti-YAP (#4912, Cell Signaling Technology) at a dilution of 1:100 as described in section 2.2.10.2. For descriptive purposes, the expression of YAP-protein, as cytoplasmic and nuclear IR in the normal thyroid tissues was compared with that in the non-neoplastic lesions i.e., inflammatory (thyroiditis) and hyperplastic lesions in section 3.6.1.1; and in the neoplastic lesions including benign tumours (follicular adenoma) and malignant neoplastic lesions (different thyroid cancers) in section 3.6.1.2.

3.6.1.1 Expression of total YAP-protein in non-neoplastic thyroid lesions

3.6.1.1.1 Cytoplasmic expression of total YAP-protein in non-neoplastic thyroid lesions

In each type of non-neoplastic thyroid lesion, the cases were grouped according to their cytoplasmic IR of YAP-protein into 4 grades: negative; low; moderate or high grade, and finally the number (%) of cases in each grade was counted in each type of lesion to compare with that in the normal thyroid tissues (Figure 3-12).

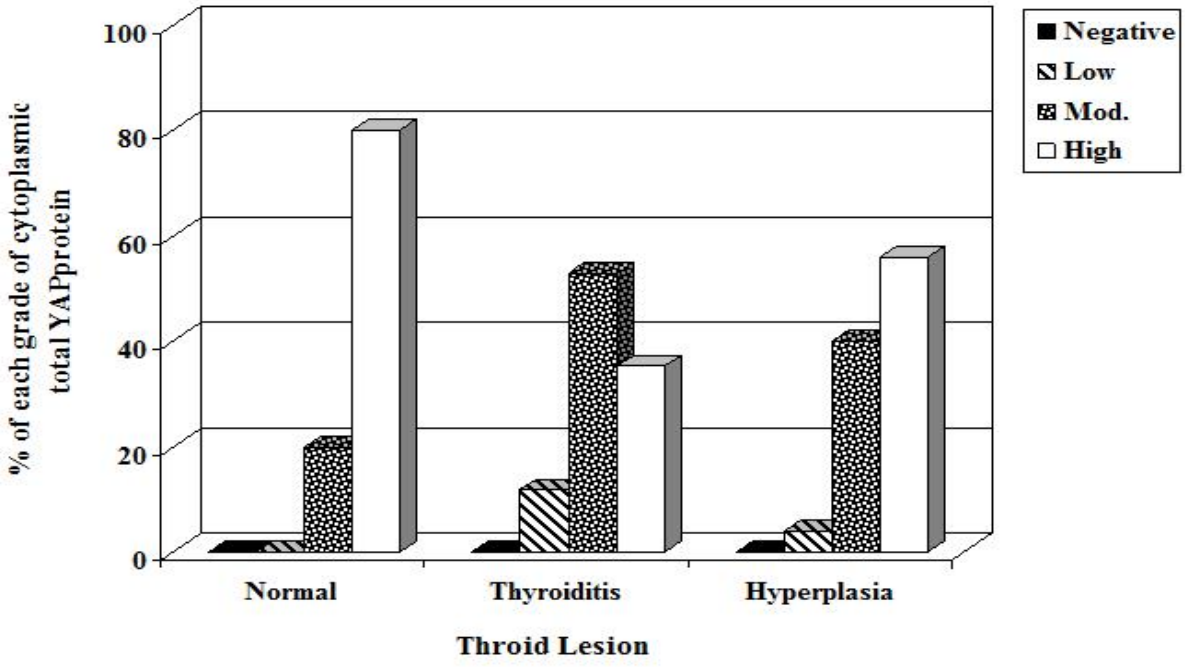

Figure 3-12: The number (%) of the cases in relation to the cytoplasmic IR of YAP-protein in different types of the non-neoplastic thyroid lesions.

The figure shows that all cases of the normal thyroid tissues express YAP-protein in their cytoplasm, and up to 80% of them are of high grade while the remaining are of moderate grade. At the same time, YAP-protein was also expressed by the cytoplasm of all cases of thyroiditis and hyperplasia. However, the proportion of those with high grade becomes lower in hyperplasia and less in the thyroiditis (56% and 35.29% respectively), where the majority of the remaining cases are of moderate grades (40% and 52.94% respectively).

In order to assess whether the expression of cytoplasmic YAP-protein in the non-neoplastic lesions changes from that in the normal thyroid tissue regarding its intensity as well the proportion of positive cells, the mean of cytoplasmic IR of YAP-protein in each group of the non-neoplastic thyroid lesions was compared with that of the normal thyroid tissues (Figure 3-13).

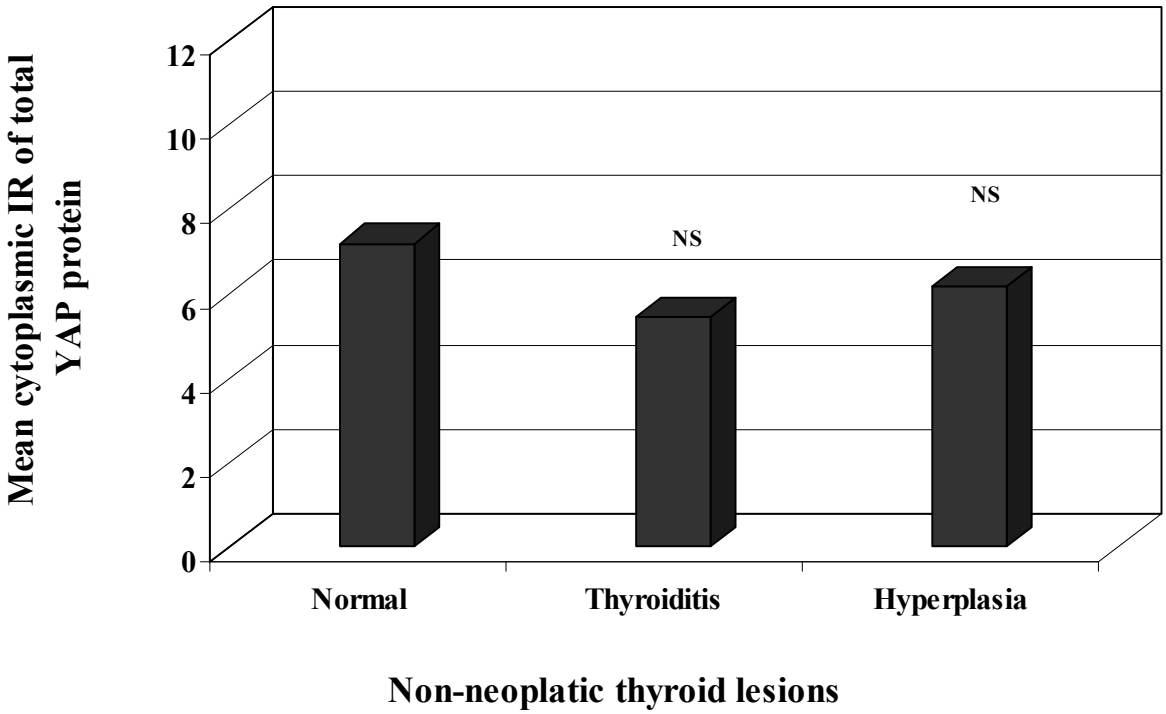

Figure 3-13: Correlation of the mean of cytoplasmic IR in different non-neoplastic thyroid lesions to that in the normal thyroid tissue (NS: *P-value* >0.05).

It shows that the mean of cytoplasmic expression of YAP-protein in normal thyroid tissues is of a moderate grade (7.2). In the non-neoplastic lesions, the mean is nearly the same in thyroiditis (5.7), but slightly increased in hyperplasia (6.2). However, these changes are not associated with any statistically significant difference (*P-value* > 0.05).

3.6.1.1.2 Nuclear expression of total YAP-protein in non-neoplastic thyroid lesions

As with the cytoplasmic YAP-protein in the section (3.6.1.1 1), the number (%) of cases in each grade of nuclear IR of YAP-protein in each type of non-neoplastic thyroid lesions was counted and compared with that of normal thyroid tissues (Figure 3-14).

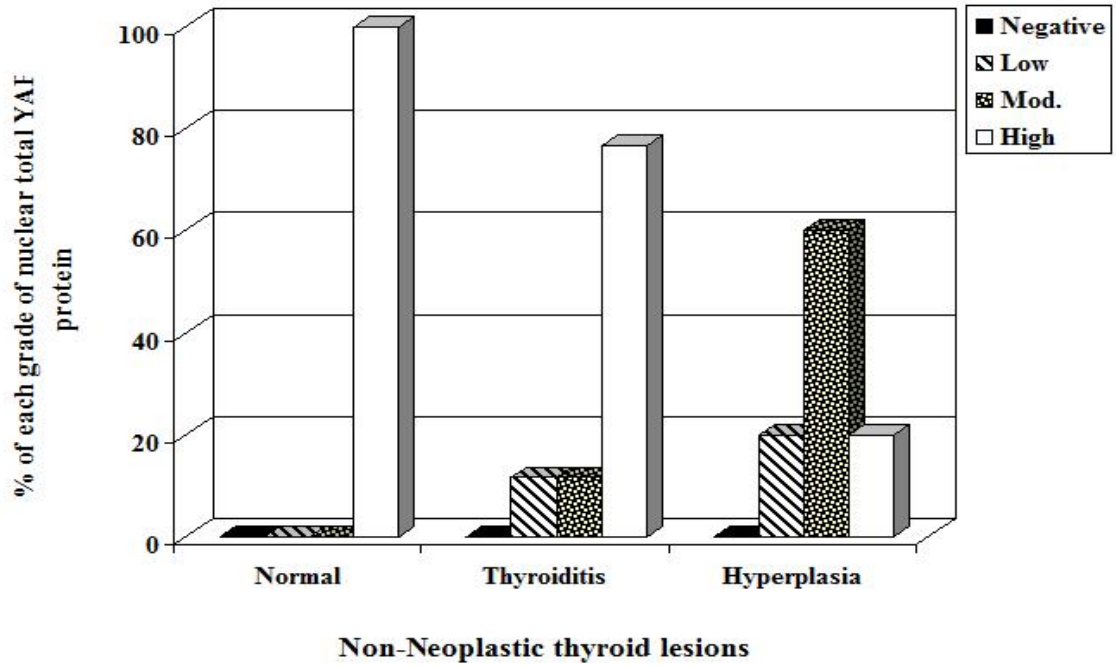

Figure 3-14: The number (%) of cases in relation to the nuclear IR of YAP-protein in different types of non-neoplastic thyroid lesions.

The figure shows that YAP-protein is expressed in the nuclei of all the cases (100%) of normal thyroid tissue with a high grade. In the non-neoplastic thyroid lesions, all the cases expressed YAP-protein in their nuclei (100%). However, the proportion of high grade becomes lower in thyroiditis (76.6%) and lowest in hyperplasia only (20%). But the majority of the remaining cases are of a moderate grade (11.7% in thyroiditis and 60% in hyperplasia).

As with the cytoplasmic YAP-protein, and in order to assess whether the expression of nuclear YAP-protein in the non-neoplastic lesions changes from that in the normal thyroid tissues, the mean of nuclear IR of YAP-protein in each type of non-neoplastic thyroid lesions was compared to that of the normal thyroid tissues (Figure 3-15).

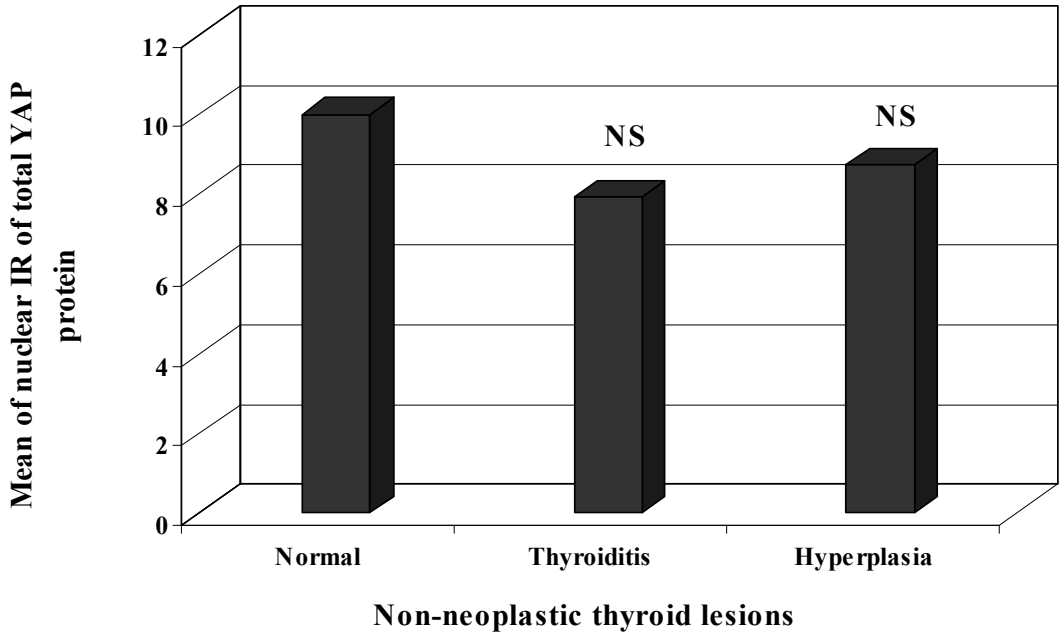

Figure 3-15: Correlation of the mean of nuclear IR in different non-neoplastic thyroid lesions with that in the normal thyroid tissues (NS:not significant difference *P-value* > 0.05).

The figure shows that the nuclear IR of YAP-protein is of high grade (10) in the normal thyroid tissues. But in both groups of non-neoplastic lesion, the nuclear IR is slightly reduced, where it becomes 7.94 in thyroiditis and 8.72 in hyperplasia. However, statistically such differences have no significance in comparison to the normal thyroid tissues (*P-value* > 0.05).

3.6.1.2 Expression of total YAP-protein in neoplastic thyroid lesions

3.6.1.2.1 Cytoplasmic expression of total YAP-protein in neoplastic thyroid lesions

In each type of neoplastic thyroid lesion, the number (%) of cases in each grade of cytoplasmic IR of YAP-protein was counted and compared to that in the normal thyroid tissues (Figure 3-16).

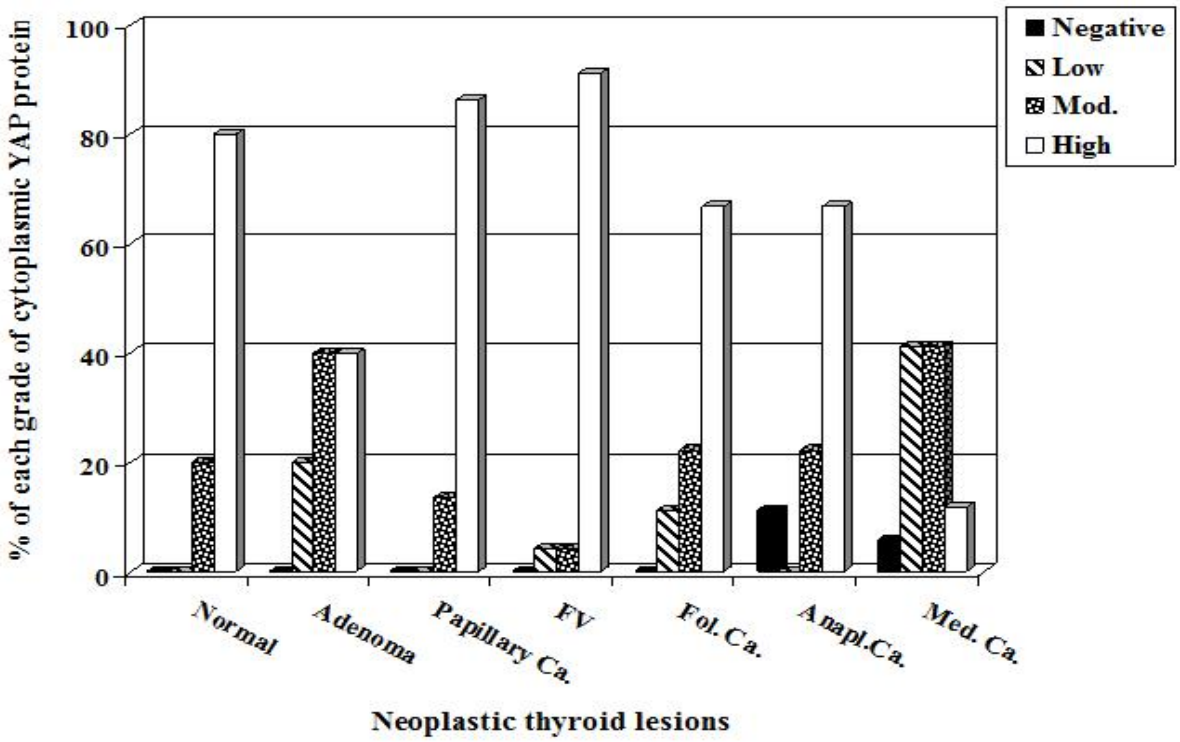

Figure 3-16: The number (%) of the cases in relation to the cytoplasmic IR of YAP-protein in different types of neoplastic thyroid lesions.

In comparison to the normal thyroid tissues, the benign neoplastic thyroid lesions (i.e. follicular adenoma) express either high or moderate grade cytoplasmic YAP-protein in the majority of cases (40% for each). At the same time, in all types of malignant neoplastic lesions, except medullary carcinoma, the cytoplasmic YAP-protein maintains its high expression as in the normal tissues in the majority of cases. In medullary carcinoma, the majority of cases show either low or moderate grade (41.1% for each).

To demonstrate the changing in the cytoplasmic expression of YAP-protein in different neoplastic lesions from that of the normal thyroid tissue, the mean of cytoplasmic IR of YAP-protein in each type of neoplastic lesion was compared with that of the normal thyroid tissues (Figure 3-17).

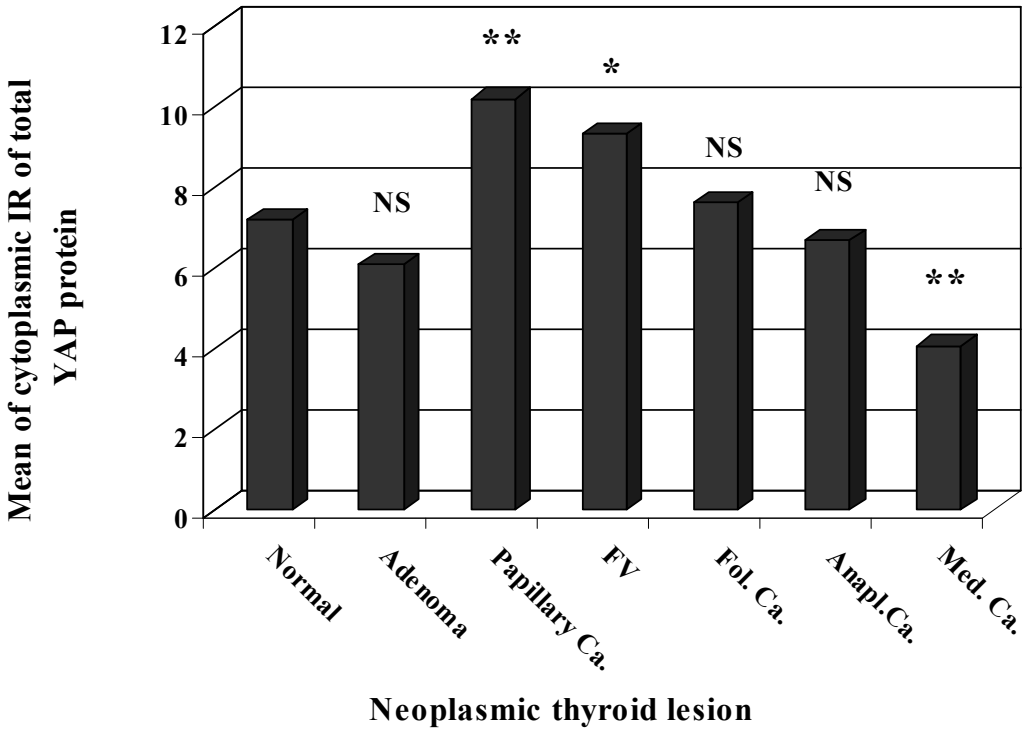

Figure 3-17: Correlation of the mean of cytoplasmic IR in different neoplastic thyroid lesions with that in the normal thyroid tissue (NS:not significant *P-value* > 0.05, *:significant difference *P-value* < 0.05, **: highly significant difference *P-value*<0.01).

It shows that the mean of the cytoplasmic IR of YAP-protein in adenomas (6.08) is slightly lower than that in the normal thyroid tissues (7.2). However, this difference statistically is not significant (*P-value* > 0.05).

The follicular carcinoma and the anaplastic carcinoma maintain the mean of cytoplasmic IR of YAP-protein at a level nearly identical to that in the normal thyroid tissues (7.61 and 6.67 respectively). Therefore, no significant statistical difference could be detected in comparison to the normal thyroid tissues (*P-value* > 0.05).

Meanwhile, in both variants of papillary carcinoma, the mean of the cytoplasmic IR of YAP-protein is significantly increased up to 10.18 in conventional variant, and up to 9.3 in the follicu-

lar variant. Statistically, such difference is highly significant in the conventional variant (*P-value* < 0.01), and significant in the follicular variant (*P-value* < 0.05)

In contrast, the cytoplasmic IR of YAP-protein in medullary carcinoma is highly reduced down to (4.06). Statistically, such difference is highly significant (*P-value* < 0.01).

3.6.1.2.2 Nuclear expression of total YAP-protein in neoplastic thyroid lesions

Figure 3-18 shows the distribution of the cases in each group of neoplastic lesions according to the grade of nuclear IR of YAP-protein in different thyroid neoplastic lesions in comparison to the normal thyroid tissues.

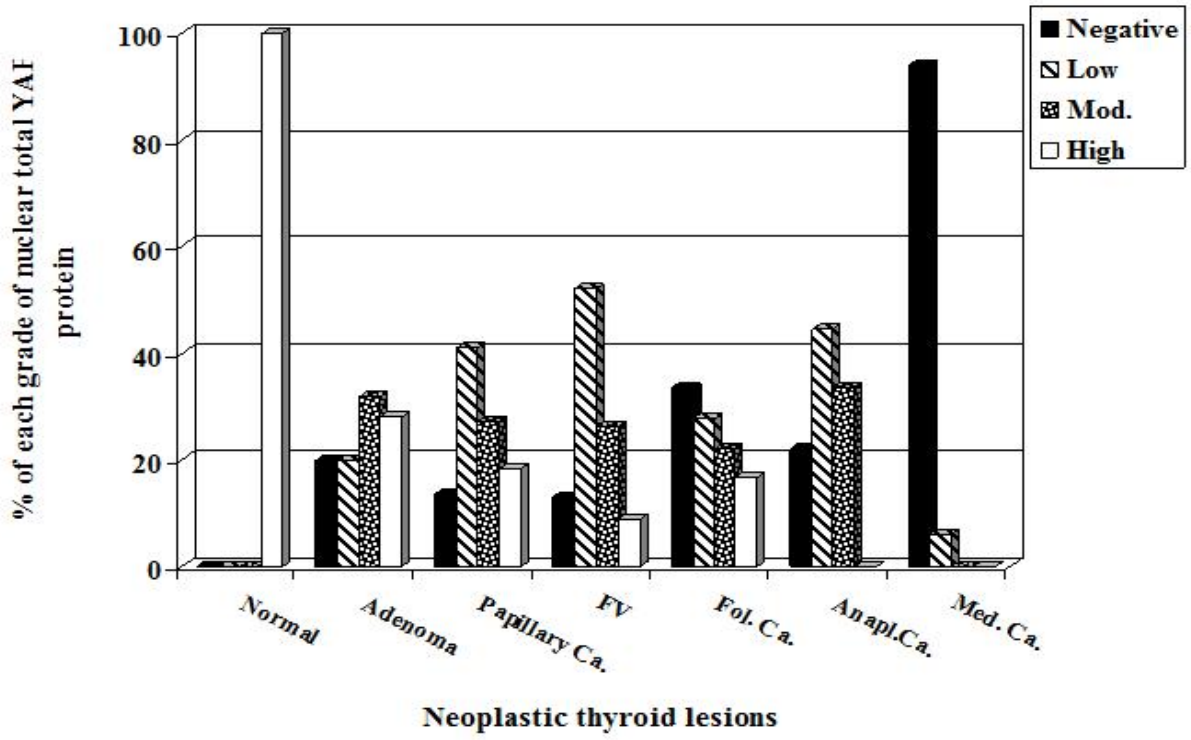

Figure 3-18: The number (%) of cases in relation to the nuclear IR of YAP-protein in different types of neoplastic thyroid lesions.

Generally, and in comparison to the normal thyroid tissues, all types of neoplastic lesions show dramatic reduction in the number (%) of cases of high grade nuclear IR of YAP-protein simultaneously with an increasing number (%) of cases with negative nuclear staining.

In benign neoplasia (i.e., adenomas), papillary carcinoma and follicular carcinomas, the number (%) of cases with a high nuclear YAP decreased dramatically from 100% in the normal thyroid tissues down to (28%, 18.18%, 8.7% and 16.67% respectively). Moreover, in anaplastic and medullary carcinomas, high grade nuclear IR of YAP-protein was undetected in any case.

On the other hand, the number (%) of the cases with negative nuclear expression of YAP-protein was significantly increased up to (20%) in adenoma, (13.64% and 13.04) in conventional variant and follicular variant of papillary carcinoma respectively, (33.33%) in follicular carcinoma, (22.22%) in anaplastic carcinoma and reached the highest level in medullary carcinoma (94.12%).

To demonstrate the change in the nuclear expression of YAP-protein in different neoplastic lesions from that of the normal thyroid tissue, the mean of nuclear IR of YAP-protein in each type of neoplastic lesion was compared with that of the normal (Figure 3-19).

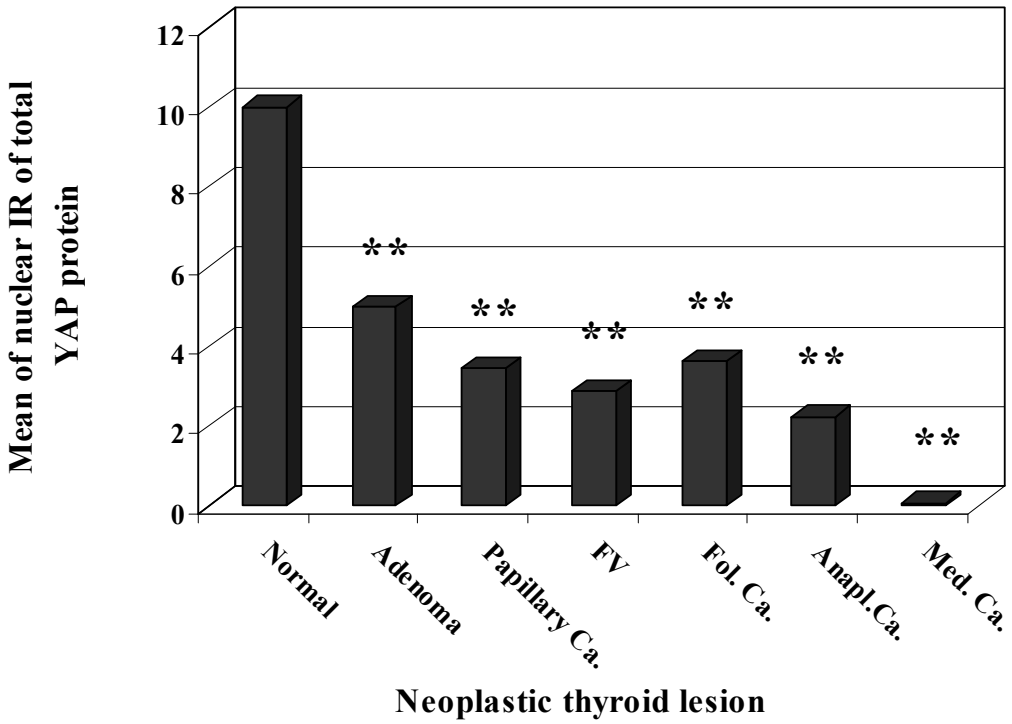

Figure 3-19: Correlation of the mean of nuclear IR in different neoplastic thyroid lesions with that in the normal thyroid tissue (: highly significant difference *P-value* < 0.01).**

It shows that the mean of nuclear IR of YAP-protein was dramatically reduced in different thyroid neoplasias, whether benign or malignant, in comparison to that in the normal thyroid tissues. Statistically, this difference is highly significant (*P-value* < 0.001).

3.6.2 Phosphorylation of cytoplasmic YAP-protein in thyroid lesions

Additional histological section from each lesion was stained immunohistochemically with anti-phospho YAP Ser-127 (#4911, Cell Signalling Technology) as described in section 2.2.10.2. This antibody is able to detect YAP-protein only when it is phosphorylated at Ser-127 residue.

3.6.2.1 Expression of cytoplasmic Phospho-YAP Ser127 in non-neoplastic lesions

The mean of IR of cytoplasmic Ph-YAP Ser-127 in each group of non-neoplastic thyroid lesions was calculated and compared with that of the normal thyroid tissues in order to demonstrate any change in the phosphorylation of YAP-protein (Figure 3-20).

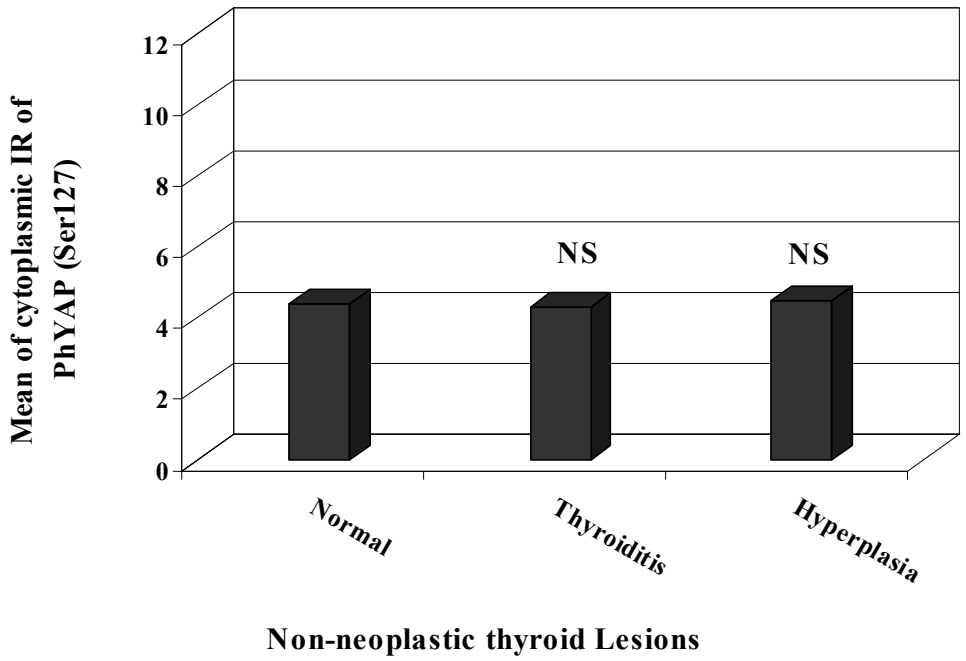

Figure 3-20: Correlation of IR of the mean of cytoplasmic Ph-YAP in different non-neoplastic thyroid lesions with that in the normal thyroid tissues (NS: *P-value* >0.05).

It shows that that the mean of cytoplasmic IR of Ph-YAP Ser-127 in the normal thyroid tissues is low (4.4). At the same time, the non-neoplastic lesions show also a nearly identical mean, where it is 4.29 in the thyroiditis and 4.48 in the hyperplasia. Therefore, no significant statistical difference could be detected in comparison to the normal thyroid tissues (*P-value*> 0.05).

In order to assess the extent of the phosphorylation of cytoplasmic YAP-protein, the ratio of IR of cytoplasmic Ph-YAP to the IR of the total cytoplasmic YAP was calculated for each case in each type of the non-neoplastic thyroid lesion. Finally, the mean of ratios in each type of non-neoplastic thyroid lesion was compared to that in the normal thyroid tissues (Figure 3-21).

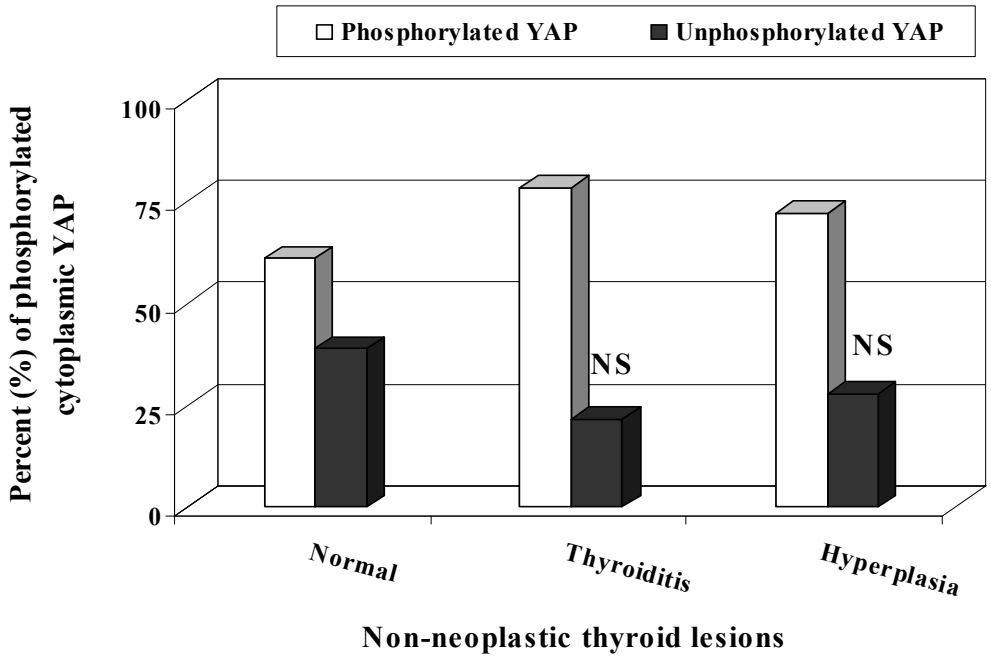

Figure 3-21: The proportion of phosphorylated YAP Ser-127 to the total cytoplasmic YAP in non-neoplastic thyroid lesions (NS: *P-value* > 0.05).

It shows that (61.1%) of the cytoplasmic YAP-protein in the normal thyroid tissues is phosphorylated at Ser-127. This ratio is slightly increased in both types of the non-neoplastic thyroid lesions, where it is reached 78.4% in the thyroiditis and 72.2% in the hyperplasia. Statistically, this increment has no significant difference in either of them in comparison to the normal thyroid tissues (*P-value*> 0.05)

3.6.2.2 Expression of cytoplasmic Phospho-YAP Ser127 in neoplastic lesions

As in the non-neoplastic lesions, the mean of nuclear IR of Ph-YAP Ser-127 in each group of neoplastic lesions was calculated and compared to that of the normal thyroid tissues in order to show the change in the phosphorylation of YAP (Figure 3-22).

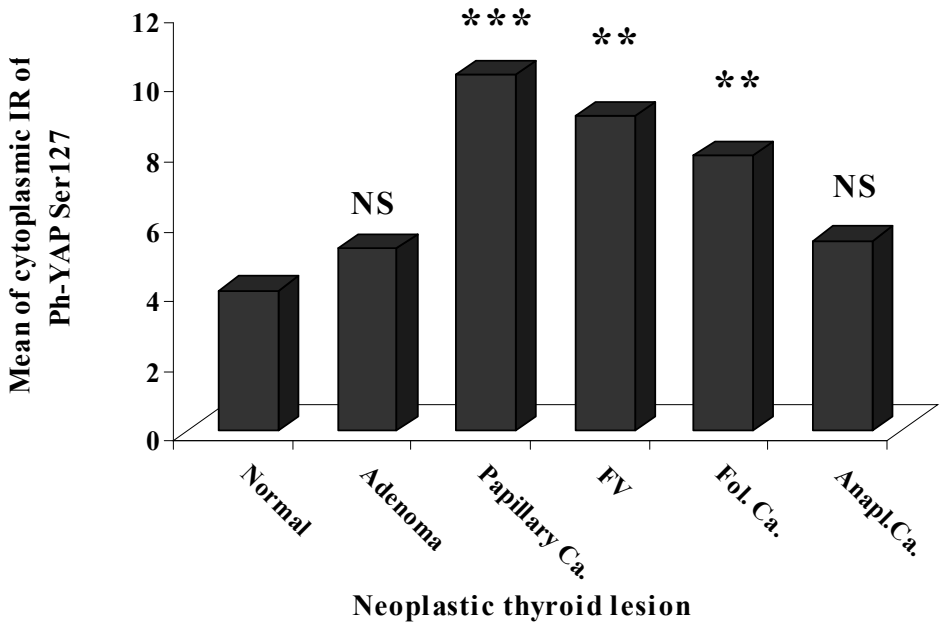

Figure 3-22: Correlation of the mean of cytoplasmic IR of Ph-YAP in different neoplastic thyroid lesions to that in the normal thyroid tissues (NS:not significant *P-value* > 0.05, **: highly significant difference *P-value* < 0.01, *:very highly significant *P-value* < 0.001).**

It shows that in follicular adenoma (benign neoplasia), the mean of cytoplasmic IR of Ph-YAP Ser-127 is 5.2, which is slightly higher than that in the normal thyroid tissues (4). However, this difference is not significant statistically (*P-value* > 0.05).

The anaplastic carcinoma also has a mean of cytoplasmic IR of Ph-YAP Ser-127 that is nearly identical to that of the normal thyroid tissues (5.44). Statistically such difference is also not significant in comparison to the normal thyroid tissues (*P-value* > 0.05).

In contrast, both variants of papillary carcinoma as well as follicular carcinoma show an obvious increment in the mean of cytoplasmic IR of Ph-YAP, where it is duplicated in conventional vari-

ant (10.18) and in follicular variant (9) and reached 7.88 in the follicular carcinoma. Statistically, this difference is very highly significant in conventional variants of papillary carcinoma (*P-value* < 0.001), and highly significant in the follicular variants of papillary carcinoma and follicular carcinomas (*P-value* < 0.01).

To demonstrate the extent of phosphorylation of cytoplasmic YAP-protein in different neoplastic lesion, the ratio of the mean of IR of Ph-YAP Ser-127 to the IR of the total cytoplasmic YAP was calculated for each lesion. Finally, the mean of the ratios in each type of neoplastic thyroid lesion was compared to that of the normal thyroid tissues (Figure 3.23).

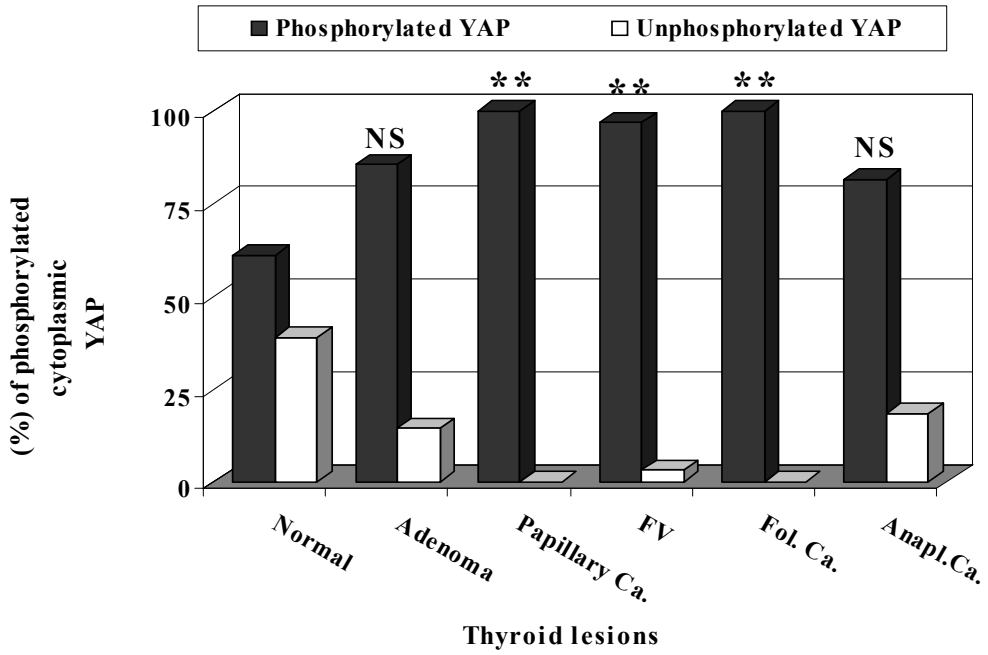

Figure 3-23: The proportion of phosphorylated YAP Ser-127 to the total cytoplasmic YAP in the neoplastic thyroid lesions (NS: not significant *P-value* > 0.05, **: high significant difference*P-value* < 0.01).

It shows that the mean of the proportion of the Ph-YAP ser-127 in different neoplastic thyroid lesions is higher than that of the normal, where it is increased from 61% in the normal thyroid tissues up to 86% in adenoma, 100% in conventional papillary carcinoma, 97% in follicular variant of papillary carcinoma, 100% in follicular carcinoma, 82% in anaplastic carcinoma. Statistically, this increment is highly significant (*P-value* < 0.01) in all of them except follicular adenoma and anaplastic carcinomas (*P-value* > 0.05).

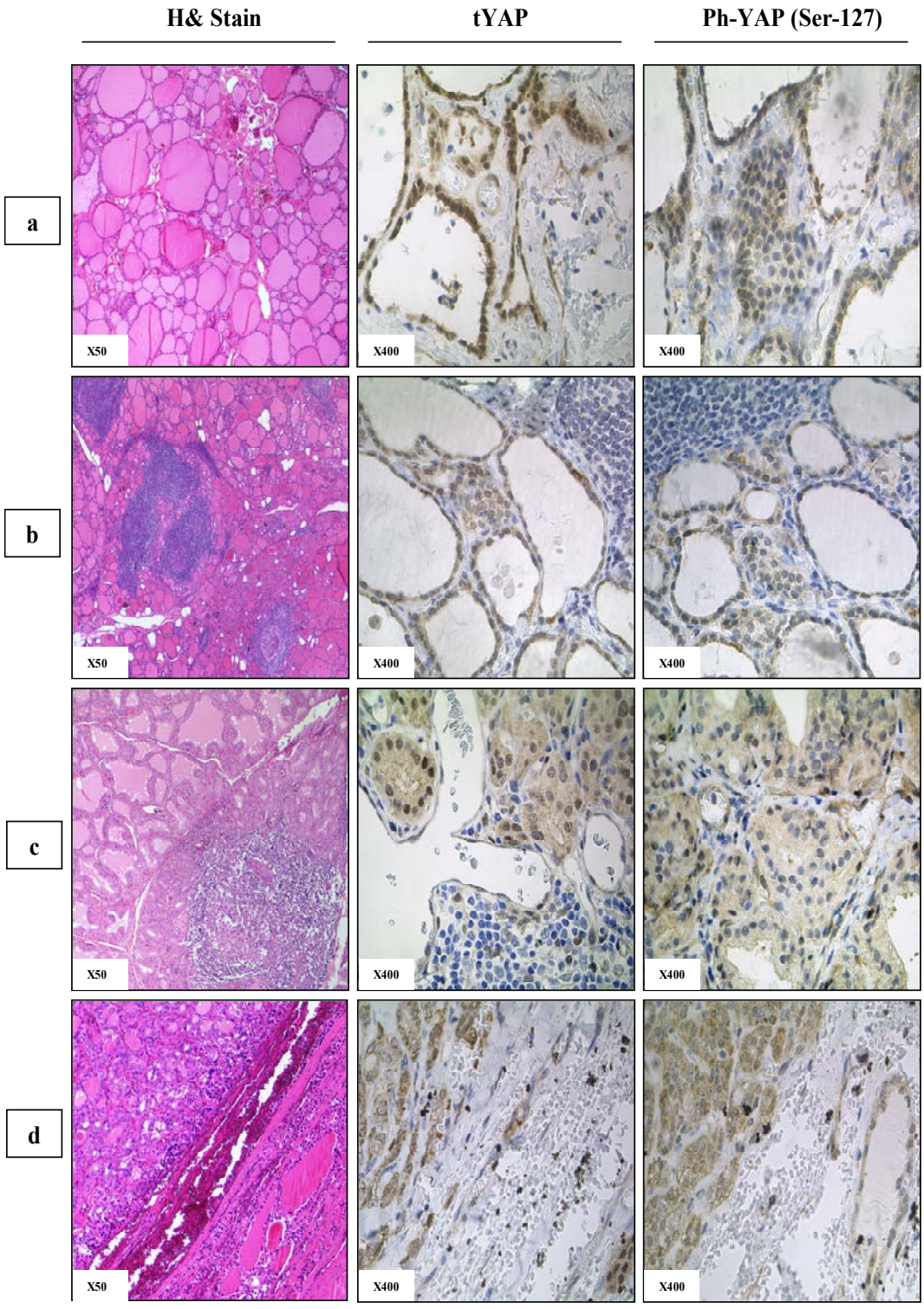

Figure 3-24: Photograph of H&E stain and immunostaining with YAP and Ph-YAP (Ser-127) in the normal and non-malignant neoplastic thyroid lesions: (a) Normal thyroid tissue, (b) Thyroiditis, (c) Hyperplasia, (d) Follicular adenoma.

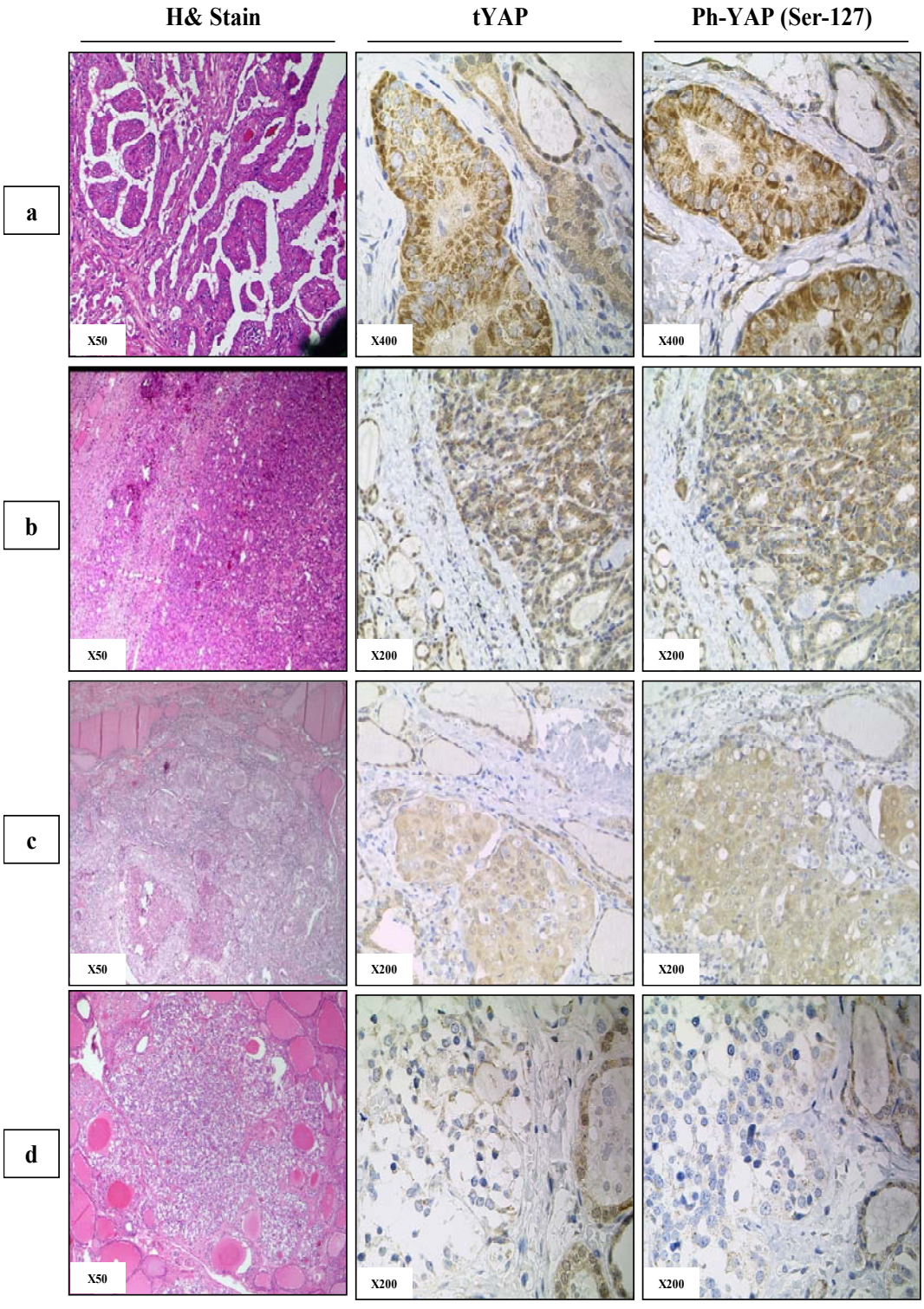

Figure 3-25: Photograph of H&E stain and immunostaining with YAP and Ph-YAP (Ser-127) in the malignant thyroid lesions: (a) Papillary carcinoma, (b) Follicular carcinoma, (c) Anaplastic carcinoma, (d) Medullary carcinoma.

4 Discussion

Recently, it was found that some proteins represent an essential component in a well- defined signalling pathway and are quite often seen to have a relation with other proteins that represent an integral component of other signalling pathways. These signalling pathways lead eventually to the most extreme and different outcomes: cell proliferation/transformation and tumour suppression/cell death (Bertini et al. 2009).

YAP-protein is an adaptor and transcription coactivator protein that has a molecular structure which enables it to bind a broad spectrum of transcription factors (Yagi et al. 1999). These factors represent the downstream of different signaling pathways. The outcomes of these pathways are quite different, where some of them promote cell proliferation and inhibit cell death, other inhibit cell proliferation and promote cell death and third control cell differentiation (Bertini et al. 2009). Accordingly, it is believed nowadays that YAP-protein may play a leading role in controlling human tumorigenesis or tumour progression. Therefore, its expression as well its role in tumorigenesis was investigated in different human organs but not yet in the thyroid. This study is an initial attempt to show the expression as well to demonstrate whether this mysterious protein has any role in human thyroid carcinogenesis or not.

Because of the lack of information about the expression of YAP-protein in the human thyroid gland, this study was designed to start with *in vitro* assessment of the expression of YAP-protein in a representative set of thyroid cancer cell lines including different types of follicular cell derived thyroid carcinomas: one cell line papillary carcinoma (B-CPAP), two cell lines of follicular carcinoma (FTC-133 and ML-1) and tow cell lines of anaplastic carcinoma (HTH-74 and C-643). The study started with an assessment of expression of YAP-protein in these cell lines compared with that in the normal thyroid tissues to demonstrate any significant change in the expression of YAP in cancerous thyrocytes from that in normal thyrocytes.

Total RNA from these cell lines and commercially available total RNA from normal human thyroid tissues (Clontech) were assessed for expression of YAP mRNA using RT-PCR. We found that the expression of YAP mRNA in all thyroid cancer cell lines particularly in FTC-133, HTH-74 and C-643 is significantly increased in comparison to the normal thyroid tissues (Figure

3-2). In addition, this expression was confirmed by immunoblotting the total protein extract from these cells lines (Figure 3-3a). Such significant difference gave us an initial clue that YAP-protein may have a role in thyroid cancer, and it encouraged us to go further to confirm these findings and to determine its biological role in thyroid cancer cells.

Accordingly, we reviewed the previous literature that assessed the biological role of YAP-protein in other human organs and we found that all of them are in absolute agreement in description of YAP-protein as a transcription factor coactivator, and it needs to localize in the nucleus of the cells to perform this function. At the same time, such absolute agreement changes to absolute controversy about the exact biological function of the transcription factors that are coactivated by YAP-protein in the nucleus. The majority of the researchers found that YAP-protein functions as an oncogenic protein by coactivating TEAD transcription factors (Zhao et al. 2008, Vassilev et al. 2001). Others found YAP-protein an apoptotic protein by coactivating p73 transcription factor or other members of the p53 family (Levy et al. 2007, Strano et al. 2005, Matallanas et al. 2007). Finally, all of them demonstrated also that phosphorylation of YAP-protein at Ser-127 is a very important determinant of its biological function because it determines the subcellular localization of this protein. In this regard, the first group who considered YAP-protein as an oncogene proposed that nuclear translocation of YAP-protein in response to oncogenic stimuli will activate TEAD-mediated cell proliferation and inhibit apoptosis (Figure 1-4) (Vassilev et al. 2001). This function is abolished by Hippo pathway where its activation will end by activation of LATS1/2 to phosphorylate the downstream effecter YAP-protein at Ser-127. This phosphorylation will result from creation of an interaction site on YAP-protein to bind in a high affinity the cytoplasmic 14.3.3 protein family that mediates its cytoplasmic sequestration and inhibits its nuclear translocation (Figure 1-5) (Camargo et al. 2007, Dong et al. 2007, Zhao et al. 2007). Therefore, phosphorylation of YAP-protein at Ser 127 is abolished in cancer cells and most of YAP-protein will be unphosphorylated and localized in the nuclei.

The other group that considered YAP-protein as an apoptotic protein suggested a completely different scenario for the mechanism and the function of phosphorylation of YAP-protein at the Ser-127 residue. They suggested that the nuclear function of YAP-protein to coactivate the apoptotic transcription factors is inhibited by its phosphorylation by the oncogenic protein AKT at the Ser-127 residue. AKT creates an interaction site on YAP-protein to bind the cytoplasmic proteins of 14.3.3 family (Figure 1-2) (Basu et al. 2003). In addition, Oka et al. 2008 reconstituted

recently the Hippo pathway in a human epithelial cell line. They established that in contrast to *Drosophila*, activation of the Hippo pathway in human results in anti-apoptotic signals. They have shown that in human epithelial cells, LATS kinases cooperate with the upstream MST2 kinases to phosphorylate YAP-proteins at Ser-127 to rescue the cells from apoptotic death. Therefore, phosphorylation of YAP-protein at Ser-127 should be increased in cancerous cells to be sequestered cytoplasmically out of the nuclei.

According to above, the extent of YAP phosphorylation at Ser-127 as well as its coexpression with its phosphorylating candidates (AKT and LATS) can act as a mirror to the biological role of YAP in human cells whether a proapoptotic or oncogenic. Therefore, the total protein from different thyroid cancer cell lines, were immunoblotted with anti- PhosphoYAP Ser-127, Phospho-AKT Ser-374 and LATS1 (Figure 3-3 b, c & d, respectively).

We found that the band of Ph-YAP Ser-127 (Figure 3-3 b) and in comparison to the bands of the total YAP-protein (Figure 3.3 a), are nearly identical regarding their thickness and intensity in all of the cell lines. This gives a suggestion, that the majority of YAP-protein in these cell lines has been already phosphorylated at Ser-127 residue. Western blot showed also that in all thyroid cancer cell lines, phosphorylation of YAP-protein Ser-127 is associated with a strong coexpression of only AKT in follicular carcinoma cell lines, only LATS1 in papillary carcinoma cell line or with both of them in anaplastic carcinoma cell lines (Figure 3-3 c and d, respectively). These findings are consistent with the findings of others regarding AKT expression in thyroid gland but not LATS1, because the latter had never been investigated in thyroid gland to date. Matthew (2001) assessed at first the expression of AKT protein in follicular and papillary thyroid cancers. He found the expression of AKT as a protein and mRNA is increased only in follicular carcinoma but not in papillary carcinoma in comparison to the normal thyroid tissues. Later Hou et al. (2008) investigated the overall occurrence and relationship of genetic alterations in the PI3K/AKT pathway in thyroid tumours. Their data provided a strong genetic implication that aberrant activation of PI3K/AKT pathway plays an extensive role in thyroid tumorigenesis particularly in follicular and anaplastic carcinomas, and promotes progression of benign thyroid adenoma to follicular carcinoma and to anaplastic carcinoma as the genetic alterations of this pathway accumulate. Vasko & Saji (2007) assessed also the expression of AKT-protein immunohistochemically in different types of thyroid tumours to assess its expression as well as its subcellular localization. They demonstrated that activation of AKT, particularly AKT1, is associated with tumour invasion and metastasis in follicular and papillary cancers, and with cellular atypia in apparently benign follicular adenomas. Accordingly, genetic alterations of the

PIK3/AKT pathway have an interesting and oncogenically important distribution pattern in different types of thyroid cancer. These genetic alterations were far more common in follicular thyroid carcinoma than in papillary thyroid carcinoma.

Our findings demonstrate that in all types of malignant thyroid cells, the majority of YAP-protein is phosphorylated at Ser-127 and it is associated with coexpression of either AKT &/or LATS1. Taking in a context these findings with the discussion above, we can say that these findings are in complete agreement with authors who considered YAP as an apoptotic protein and in contradiction to those who considered YAP as an oncogenic protein.

Since the cancer in the thyroid gland as in other human organs results simply from dysregulation of the balance between two equal but opposite forces in a human cell: proliferation and apoptosis. Accordingly, and in order to confirm our findings, and to demonstrate the real biological role of YAP-protein in thyroid cancer, we assessed the correlation of this protein with these two processes in thyroid cancer cells. For this purpose, one thyroid cancer cell line was selected for such assessment.

The principle of this assessment was based on the selection of a thyroid cancer cell line that expresses an increased level of YAP mRNA in comparison to the normal thyroid tissues as well as a strong coexpression of Ph-YAP protein Ser127 with a high level of activated AKT. In this cell line, YAP-protein expression was knocked-down by RNA-interference technique, and then we assessed the rate of cell proliferation and apoptosis before and after the downregulation to demonstrate experimentally the exact effect of YAP-protein on these two processes in a human thyroid cancer cell, and in turn its effect on the biological behaviour of the thyroid cancer cell.

FTC-133 cell line was selected, because this cell line fulfils all the above mentioned criteria regarding YAP mRNA and protein expression and its phosphorylation at Ser-127 (Figure 3-2 and Figure 3-3 a,b and c, respectively). YAP-protein expression was stably knocked-down in a subset of these cells by transfection with anti-YAP shRNA. Simultaneously, another subset from these cells was transfected with an empty vector as a negative control for transfection as described in section 2.2.6.6. Finally, a monoclone from each transfection was selected. In the monoclone with anti-YAP shRNA, YAP mRNA was knocked down to 20.5% of its expression in the parental cells, while the cells of monoclone of empty vector maintained 94.25% as that in the parental cells as seen in Figure 3-4. The knocking down of YAP-protein is confirmed by western blotting of total protein extract from these cells (Figure 3-5).

In this set of three clones (parental FTC-133, monoclone with anti-YAP shRNA and monoclone of empty vector), the rate of cell proliferation and apoptosis was assessed to show the correlation of the knocking down of YAP-protein on the two fundamental biological processes of the malignant cells (cell proliferation and apoptosis), and in turns to demonstrate the real biological role of YAP-protein in this type of thyroid cancer cells.

Regarding the rate of cell proliferation, we found that the doubling time of cells with downregulated YAP-protein is lower than that in the parental cells and in the cells of monoclone of empty vector (Figures 3-6, 3-7).

This result explains the contradiction in our findings in RT-PCR from that in Western Blot. The latter showed that the majority of YAP-protein in these cell lines is phosphorylated at Ser-127 and is associated with a high co-expression of either AKT or LATS1. This suggests a tumour suppressor function of YAP-protein, its tumour suppressor function being aborted in the cancerous cells by its phosphorylation at Ser-127. Accordingly, and contrary to what is well known that the tumour suppressor proteins are either downregulated or inactivated in cancer cell, RT-PCR in this study showed that the expression of YAP mRNA was increased in all thyroid cancer cell lines in comparison to the normal thyroid tissues. However, the recent findings of Aylon et al. 2010 and Vignneron et al. 2010 can be used to explain such contradiction and to confirm that our findings are consistent and as described in the followings:

Aylon et al. (2010) were the first researchers, who reported a biological function for cytoplasmic YAP-protein (Ph-YAP Ser-127), where before that it was believed that cytoplasmic YAP-protein has no function other than inactivation of the nuclear oncogenic or apoptotic function of YAP. They reported that YAP-protein has an oncogenic function in the cytoplasm, where it interferes with the formation of the proapoptotic complex LATS2/ASPP1 in response to oncogenic stresses, and abolishes in turn the translocation of this complex to the nucleus to induce LATS2/ASPP1 dependent p53 mediated apoptosis and cell death. Furthermore, Vigneron et al. (2010) strength this suggestion about the oncogenic function of cytoplasmic YAP-protein by its correlation with ASPP1, where they identified that YAP-dependent cytoplasmic retention of ASPP1 will convert the function of the latter (i.e., ASPP1) from a proapoptotic protein to an oncogenic protein and augment malignant cell properties.

Regarding the rate of apoptosis, apoptosis was induced in the cells of the transfected clones and the parental FTC-133 cell line by incubation of the cells with doxorubicin and cisplatin each

time. We selected these two reagents, because they represent the drug of choice in the treatment of advanced or disseminated thyroid cancer in clinical practice.

We found that the rate of apoptosis that is induced by cisplatin is markedly reduced after knocking down of YAP-protein, and this reduction was highly significant statistically in comparison to that in the parental cells (*P-value* < 0.01) (Figure 3-8). In contrast, the knocking down of YAP-protein showed no significant effect on the rate of apoptosis of the same cells with doxorubicin (*P-value* > 0.05) (Figure 3-9).

To confirm these findings and to exclude the non-specific effects of the DNA-plasmid transfection on the biological behaviour of the transfected cells, the cell cytotoxicity assay was assessed for the same cells separately using these two cytotoxic drugs in addition to other three cytotoxic drugs. The rate of survived cells in the anti-YAP shRNA monoclone was also compared with that of the parental cells and of the empty vector with each cytotoxic drug separately (Figures 3-10 and 3-11). We found that the knocking down of YAP-protein was associated with an increasing resistance of thyroid cancer cells to the treatment with cisplatin (Figures 3-10 a), where the IC50 increased up to 2.3 fold that in the parental cells, and statistically this difference was highly significant (*P-value* < 0.01) (Figure 3-10 b). In contrast, such effect could be seen neither with doxorubicin nor with the other three cytotoxic drugs: mitoxantron, vincristin and etoposide (Figures 3-11).

These findings are in complete agreement with the findings of other researchers (Strano et al. 2005, Levy et al. 2007, Levy et al. 2008), who investigated the correlation of YAP-protein with cisplatin treatment in particular and in response to DNA-damage in general and as described in the followings:

Strano et al. (2005) were the first researchers who investigated the role of YAP-protein in response to DNA-damage and particularly to cisplatin. They reported that p73 is required for the nuclear translocation of endogenous YAP-protein in cells exposed to cisplatin, and then within the nucleus, YAP-protein requires the promyelocytic leukaemia gene (PML) and nuclear body localization to coactivate p73. They also found that YAP imparts selectivity to p73 by promoting the activation of a subset of p53 and/or p73 target promoters. Finally, the endogenous p73, YAP, and p300 proteins are concomitantly recruited onto the regulatory regions of the apoptotic target gene *p53aip1*. At the same time, Lapi et al. (2008) found that cisplatin therapy resulted in stabili-

zation of YAP-protein by inducing a PML-mediated sumoylation of YAP-protein. Levy et al. (2007) demonstrated that cisplatin treatment induces YAP-protein not only to coactivate p73, but also to stabilize it by competition with ubiquitin E3 ligase Itch to bind p73, and prevents Itch-mediated ubiquitination of p73. This leads eventually to nuclear accumulation of p73 and induction of apoptosis. One year later, Levy et al. (2008) described how cisplatin can induce apoptosis in a human cell and the role of YAP-protein in this process. They showed that DNA-damage that induced by cisplatin, will activate directly c-ABL, and YAP-protein is the first target of the activated c-ABL, where it is phosphorylated by activated c-ABL at position Tyr357. Simultaneously, the activated c-ABL will stabilize YAP-protein. Eventually, the activated, phosphorylated and stabilized YAP-protein will have a high affinity to bind p73 and is selectively recruited onto the *bax* promoter to induce apoptosis

As shown above, cisplatin exerts its cytotoxic effects through YAP-dependent activation of p73 protein. Unfortunately, only a limited number of researches have investigated the expression and the role of this protein (i.e., p73) in thyroid carcinogenesis, although this protein was extensively studied in various types of human cancer and its role was exactly described. Frasca et al. (2003) assessed the expression as well as the role of p73 in thyroid cancer. They indicated that both isoforms of p73, the active one TAp73 and its inhibitor isoform ΔNp73, are increased in expression in different types of thyroid cancers in comparison to normal thyroid tissues. However, it is not functional in response to the DAN-damaging agent doxorubicin and it fails to cause G1 arrest and/ or apoptosis. They attributed the unresponsiveness of 73- protein to the coexpression of mutated form of p53 that suppresses the function of TAp73, or to the coexpression of the dominant negative isoform ΔNp73 that inhibits both of the wild-p53 as well as the TAp73.

Vella et al. (2009) confirmed the oncogenic function of ΔNp73 by demonstrating its ability to down-regulate PTEN levels in thyroid cancer cells directly and in a p53 independent manner. As a consequence, this will result in loss of inhibitory effect of PTEN on PI3/AKT pathway and reduction of p53 protein levels and increased cell proliferation and resistance to apoptosis of thyroid cancer cells. At the same time, Malaguaranera et al. (2008) confirmed the tumour suppressor function of TAp73 in thyroid cancer. They observed that in all thyroid cancers, only TAp73α isoform can synergistically cooperate with p53 in activating target genes and as a consequence, in potentiating the typical oncosuppressor function of these proteins. The mechanism of this cooperation is based on the increased p53 protein level and its stabilization by inhibition

MDM2-mediated p53 degradation via two mechanisms. First, TAp73 α inhibits p53 binding to the *mdm2* promoter: as a consequence, *mdm2* induction in response to p53 is reduced. Second, TAp73α competes with p53 for binding to the MDM2 protein.

All the above findings demonstrate that TAp73α isoform plays a significant role in thyroid cell carcinogenesis and its responsiveness to chemotherapy, particularly to cisplatin.

On the other hand, Strano et al. (2001) screened the physical connection of YAP-protein with different members and their isoforms of p53-protein family in a human cell, and they found that YAP-protein can bind only with the TAp73α, TAp73β, and TAp63α, but not with the other isoforms of p73 and p63 as well all forms of p53. They determined that this interaction involves direct association between WW domain of YAP and the PPPPY motif of p73. Additionally, they found that overexpression of YAP-protein causes an increase of TAp73α transcriptional activity.

Taking all the above into account, we suggest that YAP-protein may play the central role in the response of thyroid cancer cell to cisplatin by transmission of the apoptotic signal from the activated c-ABL to the nucleus, where it stabilizes and activates TAp73α isoform that in turn promotes cell suppression and apoptosis either directly or indirectly by activation of p53 to induce the powerful tumour-suppressor genes such as *p21/waf1, gadd45, 14-3-3, bax, puma, noxa, cd95/fas, pig3,* and *p53aip1* (Harms et al. 2004).

In an attempt to confirm our findings *in vitro*, YAP-expression was assessed in a representative set of thyroid tissue samples, including different non-neoplastic and neoplastic thyroid lesions in addition to the normal thyroid tissues. In this way, we demonstrated how the expression of this protein can change according to malignant potential of thyroid cells and tissues.

In each lesion, the expression of the total endogenous YAP-protein was assessed by using anti-YAP, which was in Western blot of the total protein extracts of the thyroid cancer cell lines. The assessment of immunostaining was performed by assessment of cytoplasmic and nuclear immunoreactivity in each lesion separately using “Remmele Scoring System” (Remmele & Stegner 1987). This is because the previous literature verified that not only the level of YAP-protein, but also its subcellular localization (i.e., cytoplasmic or nuclear) are the fundamental determinants of the biological function of YAP-protein in a human cell. Furthermore, in order to demonstrate any change in the pattern of expression of YAP-protein with the neoplastic transformation in thyroid

tissues, the cytoplasmic and nuclear IR of YAP-protein in the normal thyroid tissues was compared each time with that in different non-neoplastic and neoplastic thyroid lesions separately.

The non-neoplastic lesions included different types of thyroiditis and hyperplasia, while the neoplastic lesions were diagnosed and subclassified using the World Health Organization (WHO) histological classification of thyroid tumours (Table 1-1) (Hedinger et al. 1989). This is because this system classifies thyroid neoplasias into different histotypes according to the cell of origin, its malignant potential and their morphology. In addition, it has been shown that each histotype in this system is associated with a characteristic molecular alteration in its pathogenesis, clinical presentation and progression.

This study showed that the normal thyroid tissues express YAP-protein in the cytoplasm in a moderate grade and in the nuclei in a high grade. Such pattern of expression is highly conserved in most cases of both types of non-neoplastic lesions (i.e., thyroiditis and hyperplasias) (Figures 3-13 and 3-15). In contrast, this pattern of expression, in particular regarding YAP subnuclear localization, is dramatically deranged in the neoplastic thyroid lesions:

1. Benign thyroid tumours (i.e., follicular adenoma) maintain cytoplasmic YAP-protein at a high to moderate level similar to that found in the normal thyroid tissue in the majority of the cases (Figure 3-16), but they showed a significant reduction in nuclear localization of YAP-protein (Figure 3-19).

2. In the malignant neoplastic lesions, the expression of YAP-protein showed a significant derangement compared that in the normal thyroid tissues, and this derangement showed a significant correlation with both of the cell of origin of thyroid cancer and its degree of differentiation and as described in the following:

C-cell derived carcinoma (medullary carcinoma), showed a significant reduction in the cytoplasmic and nuclear expression of YAP-protein in comparison to the normal thyroid tissues, where 82 % of the cases have either negative or low cytoplasmic expression (41.18% for each) (Figure 3-16), and 94.1% of cases have negative nuclear expression (Figure 3-18). This may suggest that YAP-protein has no role in this type of thyroid cancer.

In follicular cell-derived thyroid cancers, YAP-protein seems to have an important role, where the nuclear expression is dramatically reduced in all of them (Figure 3-19), whereas, the cyto-

plasmic expression of YAP-protein either maintains its moderate level as in the normal thyroid tissues as has been observed in anaplastic carcinomas, or it is increased significantly as in both morphological variants of papillary carcinoma and follicular carcinoma (Figures 3-16 and 3-17).

As we described before, abolishing of the nuclear translocation of the YAP-protein to perform its tumour suppressor function and/or induction of apoptosis is achieved by its phosphorylation at Ser-127 to be sequestered in the cytoplasm. Accordingly, an additional slide from each lesion was stained with anti-phospho YAP Ser-127, because this antibody is able to detect YAP-protein only when it is phosphorylated at Ser-127 residue. The immunohistochemical staining of phYAP was assessed in each type of thyroid lesion and compared to that of the normal thyroid tissue. We found that the cytoplasmic IR of Ph-YAP Ser-127 is low in the normal thyroid tissue and in both types of the non-neoplastic thyroid lesions (thyroiditis and hyperplasia) (Figure 3-20). In contrast, the neoplastic thyroid lesions expressed increasing in the cytoplasmic IR of Ph-YAP Ser-127, where it was increased either to be of a moderate degree in benign neoplasia (follicular adenoma) and in anaplastic carcinoma, or to be of a high degree in papillary carcinoma and follicular carcinomas (Figure 3-22).

These findings confirmed our findings *in vitro*, and are consistent with previous studies that assessed *yap*-gene expression and YAP-protein in other human organs regarding the increment of their expression in malignant lesions in comparison to normal tissues. However, regarding the subcellular localization of YAP-protein, our findings are in agreement with those of other authors who believe that nuclear localization of YAP-protein is associated with coactivation of tumour suppressor genes. But they are in contradiction to the findings of others, who believe nuclear localization of YAP-protein is associated with coactivation of oncogenes in human cancers.

Ehsanian et al. (2010) found that YAP-protein functions as a tumour suppressor protein in a subset of head and neck squamous cell carcinoma, where they found an increased cytoplasmic expression of YAP-protein with a weak or negative nuclear expression in the cancerous lesions in comparison to the surrounding non-timorous tissues. Additionally, they attributed these findings to the cytoplasmic sequestration of YAP-protein which is mediated by AKT. They proposed AKT to phosphorylate YAP-protein at Ser-127 to induce its binding to the cytoplasmic 14.3.3 protein family and prevent its nuclear translocation to mediate the tumour suppressor genes.

Yuan et al. (2008) also demonstrated immunohistochemically that normal breast tissues exhibit a strong nuclear expression of YAP-protein in the myoepithelial cells with weaker cytoplasmic

expression in the luminal epithelial cells. In hyperplasia, strong nuclear YAP-protein immunoreactivity was maintained in the myoepithelial cells, while the proliferating luminal population displayed a mixed pattern of nuclear reactivity and cells negative for YAP. This is in keeping with the mixed myoepithelial/ luminal proliferation characteristic of benign hyperplasia, though it indicates early loss of luminal YAP. In ductal carcinoma in situ (DCIS), they found again that nuclear YAP was maintained in myoepithelial cells, but the transformed luminal cells were negative for YAP in 63% of the cases. In infiltrating ductal carcinoma, most of the cases showed loss of YAP expression. Therefore, they concluded that YAP-protein is a tumour suppressor protein and loss of its expression represents an early and primary event in breast tumorigenesis.

On the other hand, our findings are in complete contradiction to others who considered YAP-protein to be an oncogene regarding its subcellular localization in cancerous cells (Steinhardt et al. 2008, Le et al. 2010, Xu et al. 2009, Da et al. 2009, Muramatsu et al. 2011). Contrary to our findings, they found that the overexpressed YAP-protein in cancerous lesions is localized predominantly in the nuclei rather than predominantly cytoplasmic in the normal or non-neoplastic tissues, and accordingly, they proposed YAP-protein to be an oncogenic protein by coactivation oncogenic transcription factors to promote cell proliferation and/or inhibit cell apoptosis.

Steinhardt et al. (2008) found that YAP-protein is expressed focally in the progenator and reparative cellular compartments of the normal tissues of ovary, colon and lung. In contrast, there was a strong and diffuse nuclear and cytoplasmic, but predominantly nuclear YAP-protein expression in adenocarcinoma of these organs. They attributed these findings to the inactivation of the Hippo pathway in these cancers that in turn results in releasing YAP-protein to translocate into the nuclei and exerts its oncogenic function (Figure 1-5). Le et al. (2010) confirmed this suggestion by demonstrating the defect in Hippo pathway leads to loss of the cytoplasmic phosphorylation of YAP-protein and subsequent cytoplasmic sequestration, which eventually results in its nuclear translocation and exerting its oncogenic function. Xu et al. (2009), Da et al. (2009), Muramatsu et al. (2011) also identified YAP-protein as an oncoprotein, and demonstrated that it is overexpressed and localized predominantly in the nuclei of the neoplastic lesions cells in comparison to the normal tissues.

Putting our findings in immunohistochemistry, RT-PCR and Western blot in a context, it can be concluded that the *yap*-gene is increased in expression in follicular cell-derived thyroid cancer in comparison to the normal thyroid tissues. However, the majority of the product of the overex-

pressed gene in these cancers tend to be phosphorylated at Ser-127 and localized predominantly in the cytoplasm of the neoplastic cells and not in their nuclei in opposite to the normal thyroid tissues and the non-neoplastic thyroid lesions, where YAP-protein distributes among the cytoplasm and nuclei of thyroid cells with predominant localization in the nuclei. At the same time, cytoplasmic localization of phosphorylated YAP Ser-127 has a dual effect:

1. It showed a significant association with the rate of proliferation in the thyroid cancer cell line (FTC-133), where downregulation of YAP resulted in a significant reduction in the proliferation rate of cancer cell.

2. It plays a significant role in the chemoresponsiveness of thyroid cancer cell line (FTC-133) to cisplatin but not to the other cytotoxic drugs by increasing the rate of apoptosis, where its downregulation was associated with a significant reduction in the rate of apoptosis and increasing cell survival with cisplatin but not with the other cytotoxic drugs (doxorubicin, mitoxantron, vincristin and etoposide).

5 Abstract

YAP-protein is a transcription factor coactivator which is able to coactivate a broad spectrum of transcription factors that represent the downstream of different signaling pathways leading to quite different outcomes: oncogenesis, tumour suppression and apoptosis or differentiation. This study is an attempt to assess the expression of YAP protein and its biological role in cell lines representing different types of follicular cell-derived thyroid cancer and tissue specimens of normal, non-neoplastic and different neoplastic thyroid lesions using RT-PCR, Western blot and immunohistochemistry.

RT-PCR demonstrated an increased expression of YAP mRNA in different types of thyroid cancer cell lines in comparison to that in the normal thyroid tissues. Western blot confirmed YAP mRNA expression and showed that most of the YAP protein is phosphorylated at Ser-127 and strongly coexpressed with its candidates phosphorylating kinases AKT and/or LATS1. To demonstrate the real biological function of YAP-protein in these cell lines, YAP expression was knocked down in one thyroid cancer cell line (FTC-133) by RNAi technique using anti-YAP shRNA. The knocking down led to a dramatic reduction in apoptosis simultaneously with increasing resistance to cisplatin treatment but not to other four cytotoxic drugs (doxorubicin, mitoxantron, vincristin and etoposide). Immunohistochemistry confirmed these findings, where it demonstrates that YAP protein is increased in its expression and accumulates predominantly cytoplasmically as a phosphorylated form at Ser-127 residue with a prominent reduction in its accumulation in the nuclei in different follicular cell- derived thyroid carcinomas in comparison to normal thyroid tissues.

This study concluded that the high nuclear expression of YAP in normal thyrocytes will be changed to accumulate predominantly in the cytoplasm in malignant thyrocytes. Ser-127 phosphorylation of YAP may play a significant role in this change. At the same time, YAP plays a significant role in selectively increasing chemosensitivity of thyroid cancer cell (FTC-133) to cisplatin but not to doxorubicin, mitoxantron, vincristin and etoposide.

6 Zusammenfassung

Das Yes assozierte Protein (YAP) ist ein transkriptionfaktorkoaktivator für ein breites Spektrum von Transkriptionsfaktoren, welche in eine Vielzahl von Signalwegen involviert sind, die wiederum an verschiedenen biologischen Prozessen wie Zelldifferenzierung, Tumorsuppression bzw. Onkogenese beteiligt sind.

Ziel der vorliegenden Arbeit ist die Untersuchung der YAP-Expression in follikelzell- ausgehenden Schilddrüsenkarzinomlinien und Schilddrüsengeweben verschiedener Schilddrüsenläsionen auf Protein- und Genebene, durch *real-time* PCR, Western Blot und Immunhistochemie. Ferner sollte der Einfluss von YAP auf die Proliferation, Apoptose und Chemoresistenz in malignen Schilddrüsenzellen ermittelt werden.

Auf mRNA-Ebene konnte, im Vergleich zu normalen Schilddrüsengeweben, eine erhöhte YAP-Expression in den verschiedenen Schilddrüsenkarzinomzelllinien festgestellt werden. Diese Beobachtung ließ sich auf Proteinebene, mittels Western Blot-Technik und Immunhistochemie, bestätigen. Dabei zeigte sich, dass follikelzell- ausgehende Schilddrüsenkarzinomen eine vornehmlich zytoplasmatische Akkumulation von YAP aufweisen. Überdies war der Großteil der YAP-Proteine am Serin 127 phosphoryliert. Und stark exprimiert mit den Zielprotienen AKT und/oder LATS1. Um die biologische Relevanz von YAP *in vitro* näher zu untersuchen, wurde dessen Expression in der Zelllinie FTC-133 durch den Einsatz von anti-YAP shRNA gehemmt. Die verminderte YAP-Expression hatte eine signifikante Reduktion der Apoptose, bei gleichzeitig verstärkter Resistenz gegenüber Cisplatin, aber nicht gegenüber Doxirubicin, Mitoxantron, Vincristin und Etoposid, zur Folge.

Die vorliegende Arbeit zeigt, dass maligne Thyrozyten, im Gegensatz zu normalen Thyrozyten, eine zytoplasmatische Überexpression von YAP aufweisen. Höchstwahrscheinlich ist die Phosphorylierung von YAP am Serin 127 dabei von Bedeutung. Gleichzeitig spielt YAP eine wichtige Rolle in der selektiven Erhöhung der Chemosensitivität gegenüber Cisplatin.

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8 Appendix

8.1 Index of Abbreviations

ABL	Abelson murine leukemia viral oncogene homolog 1
APS	Ammoniumperoxide sulfate
ATC	Anaplastic thyroid carcinoma
BCA	bicinochoninic
BSA	bovine serum albumin
C-cell	Calcitonine producing cells
ccnd/bcl1	cyclin D/ B-cell leukemia lymphoma 1
CO_2	carbon dioxide
Ct	cycle threshold
CTGF	connective tissue growth factor
Cu	cupper
DAB	diaminobenzidin
dH_2O	distilled water
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dsRNA	double stranded RNA
EBP50	ERM-binding protein 50 kDa
EMT	epithelial to mesenchymal transformation
FASL	FAS ligand
FCS	Fetal calf serum
FTC	Follicular thyroid carcinoma
h	hour
HRP	horse radish peroxidase
IR	immunoreactivity
ITCH	E3 ubiquitin ligase protein itchy homolog
JNK	Jun N-terminal kinase
LATS	large tumour suppressor
M	molar
MAPK	mitogen activating pathway
MDM	p53 binding protein
MEN	multiple neuroendocrine neoplasia
miRNA	micro RNA
min	minute
MOPS	3-(n-morpholino)-propanesulfonic acid
mRNA	messenger ribonucleic acid
MST	mammalian Ste20- like protein
MTC	medullary thyroid carcinoma
NCK	non-catalytic region of tyrosine kinase adaptor protein
nM	nano molar
OD	optical density
PBS	phosphate buffered saline

PDZ	binding motif **(**post-synaptic density, discs large, zonula occludens-1
P53AIP1	p53- regulated apoptosis- inducing protein 1
PEBP2	polyomavirus enhancer binding protein 2
PI	propidium iodide
PI3K	Phosphatidyl inositol tri-phosphate kinase
PML	promyelocytic leukaemia
PPARγ	peroxisome proliferation activated receptor **γ**
PS	phosphatidyl- 3- kinase
PTC	papillary thyroid carcinoma
PTEN	phosphatase and tensin homologue
RASSF1	RAS associated domain containing protein 1
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA-interference
rRNA	ribosomal RNA
RT-PCR	Reverse transcription- polymerase chain reaction
SDS	sodium dodecylsulfate
sec	seconds
ShRNA	small hairpin RNA
siRNA	small interfering RNA
SRB	sulforhodamin-B
TA	transactivation domain
TBST	tris-buffered saline
TCA	trichloroacetic acid
TEAD	transcription enhancer activation domain
TEMED	N,N,N,N-tetramethylethylenediamine
TRK	tyrosine receptor kinase
UV-Light	Ultraviolet light
WHO	world health organization
YAP	yes associated protein

8.2 Index of figures

8.3 Index of tables

8.4 Acknowledgment

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Lebenslauf

"Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht."

List of Publications

1) Derwiysh A, Al-Aubaidy S, Al-Ahmad A. Histopathological, Immunohistochemical and Serological Study of *H. Pylori* associated Chronic Gastritis. Iraqi Postgraduate Medical Journal.2006;5:312-17.

Selbständigkeitserklärung

„Ich, Alaa Derwiysh, erkläre, dass ich die vorgelegte Dissertation mit dem Thema Yes associated protein (YAP) expression and its biological role in thyroid gland selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.“

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