# 2

# The Functionality of p53 in Thyroid Cancer

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## 1. Introduction

Abnormalities in several important cellular pathways and processes are often a major contributing factor to the progression of cancer. The cell cycle is regulated by checkpoint proteins, including the cyclins and cyclin dependent protein kinases (cdk), which play an important role in the prevention of aberrant cell division. Normal cells have the ability to stop cell division and initiate DNA repair or apoptosis (programmed cell death) when genomic abnormalities cannot be repaired. Apoptotic signaling is necessary for the elimination of unwanted cells arising from exposure to stress or toxins or as a function of normal tissue development and senescence. Proteins regulating these pathways are critical to maintain genomic integrity by controlling normal cell division and cell death; one of the most important of these is the tumor suppressor p53. The p53 protein plays a critical role in maintaining genomic integrity through control of cell division, apoptosis, DNA repair and angiogenesis, and thus is known as the "guardian of the genome" (Lane, 1992). Cancer cells have evolved the ability to bypass the cell cycle checkpoints, to favor anti-apoptotic pathways and thus, proliferate uncontrollably. Many anti-tumor drugs and treatments such as radiation are designed to target the induction of pro-apoptotic pathways in cancer cells in an effort to stop cell division and ultimately kill these aberrant cells. In many cases, these drugs and treatments activate p53 which can then regulate the expression of genes controlling the cell cycle and apoptotic pathways. Understanding the role of the tumor suppressor p53 in the regulation of these processes is the focus of many laboratories around the world.

Many cancer cells have incorporated genetic alterations that allow the cells to evade the normal control of cell cycle and apoptotic processes and gain survival advantage. Oncogenic activation of the RAS/RAF/MEK/ERK pathway is considered the most common molecular alteration in thyroid cancer. Loss of function of tumor suppressors, such as PAX-8/PPARγ, PTEN, β-catenin and p53 has been observed in thyroid cancers, so these changes are likely involved in the progression of the disease (Hunt, 2005; Kroll, 2004). p53 is found to be mutated in 50% of human cancers, yet mutations in this gene are found in only 10% of thyroid cancers, primarily in poorly differentiated and aggressive types (Olivier et al., 2002). Based on these data, it is probable that the majority of thyroid cancers activate an alternative pathway that compromises the function of wild-type p53. Well-differentiated thyroid cancers generally do not express a mutation in p53 (Fagin et al., 1993). This suggests that

mutation in the *p53* gene is a late stage event in the progression of thyroid cancer. The function of mutant p53 in thyroid cancer is not well understood.

### 1.1 p53 structure and regulation

p53 is a tumor suppressor protein transcribed by the *TP53* gene. The p53 protein is composed of 393 amino acids which span 5 conserved regions, including (1) an acidic N-terminus transcription-activation domain (residues 1-75); (2) a proline-rich domain (residues 64-92); (3) a DNA-binding domain (DBD) (residues 100-292); (4) an oligomerization domain (residues 324-355); and (5) a basic C-terminal regulatory domain (residues 360-393). Also of importance are the nuclear localization signal, NLS (residues 316-325) and the nuclear export signal, NES (residues 356-362), in the oligomerization domain and the C-terminal regulatory domain, respectively. Oligomerization of each p53 monomer and subsequent dimerization is a prerequisite to the formation of functionally active tetrameric p53 protein (Jeffrey et al., 1999); therefore, the tumor suppressor activity of p53 is dependent on tetramerization of each dimeric p53 molecule. Although p53 proteins that harbor a missense mutation in the oligomerization domain are known, these mutant monomers are generally functionally inactive and therefore these are not likely to play a role in altered DNA binding specificity. The full crystal structure of the wild-type p53 protein bound to DNA has been elucidated and well analyzed, and a list of "hot spot" mutations within the DBD associated with cancer are at sites that make contact with DNA. The structural complexity of these mutations and their altered activity in DNA binding are not well understood, although studies show that mutations are frequent at certain residues (i.e. R175, R273) in the DBD and may affect thermodynamic stability of the protein, or lead to steric interference and conformational changes at the DNA binding surface (Joerger et al., 2006).

The amount of p53 in normal cells is maintained at a very low level (Collavin et al., 2010). p53 activation in response to environmental challenges results in an overall increase in the accumulation and stabilization of p53, as well as qualitative changes in the protein, culminating in regulation of p53 target genes in the cell. p53 is activated under conditions of cellular stress and binds DNA within the promoters of target genes. p53 is capable of arresting the cell cycle at G1, G2 or in S phase, largely by induction of the *p21* gene whose protein product subsequently blocks the cdk's responsible for checkpoint regulation and progression of the cell cycle (Bai & Zhu, 2006). This allows the cell adequate time to repair damage to replicating DNA, or if this damage cannot be repaired, higher levels of p53 may signal a different set of genes that induce cell death through up or down-regulation of p53 targets that control apoptosis. Through these and other mechanisms, p53 works to maintain genomic stability. Transcriptional activity of the p53 protein is highly regulated by post-translational modification (PTM), particularly phosphorylation of serine (S) residues in both the N-terminal transactivation domain and the C-terminal regulatory domain, as well as acetylation of multiple lysine (K) residues in the C-terminal regulatory domain (Xu, 2003). Acetylation at residues K372, K373, K381 and K382 has been implicated in blocking the ubiquitin ligase activity of Mdm2, while other research suggests that phosphorylation at S15 and S392 results in accumulation of p53 in cancer cells by inhibiting interaction with Mdm2 (S15) and by blocking the Mdm2-ubiquitin degradation pathway (S392) (Kubbutat et al., 1998). As p53 is an activator of the *mdm2* gene, there is a negative auto-regulatory feedback loop between p53 and Mdm2, which works to maintain low levels of p53 in normal cells.

Once activated, p53 upregulates the gene which encodes the ubiquitin ligase that induces its degradation. This feedback pathway restricts the growth inhibitory actions of p53 in unstressed cells (Kubbutat et al., 1997). When p53 loses its ability to induce the *mdm2* gene and propagate the aforementioned negative feedback loop, the p53 protein accumulates in cells. This has been associated with poor clinical outcome (Vogelstein et al., 2000). Jung and colleagues (2011) recently reported that they observed a time and dose-dependent effect when treating adenocarcinoma cells (Ishikawa cells) with hydrogen peroxide, resulting in an increase in the amount of p53 protein and an increase in Bax, a pro-apoptotic member of the *bcl2* gene family. High levels of p53 staining observed in previous immunohistochemistry (IHC) studies of biopsy samples indicate that mutant p53 accumulates in tumors (Alsner et al., 2008). Interestingly, we and others have observed an accumulation of wild-type as well as mutant p53 in the nucleus of various cancer cell lines (Chandrachud & Gal, 2009; Olivier et al., 2005; Ray & Gal, unpublished). Hence, it becomes important to understand the functional significance of the p53 found in tumor samples, irrespective of the type of cancer.

### 1.2 The role of p53 in thyroid carcinoma

The role of *TP53* mutation in anaplastic thyroid cancer has been documented through research over the years. Mutation of *TP53* has been implicated as a late event in thyroid carcinomas (Pollina et al., 1996; Blagosklonny et al., 1998). In various experiments performed thus far, only about 14% of the thyroid tumors studied harbor p53 mutations (Shehdain, 2001). Mutations in p53 are found in poorly differentiated thyroid tumors (Shehdain, 2001) as well as in thyroid tumors displaying distant metastasis (Pavelic et al., 2006). Among the benign thyroid lesions, 100% of the cases of adenomatous goiter, thyroiditis and Grave's disease in this study showed the presence of wild-type *TP53* gene, 5% of follicular adenoma tumors display p53 mutation and 5% of the Hurtle adenomas show loss of heterozygosity (LOH) of the *TP53* gene. Among the malignant thyroid tumors analyzed in one study, advanced cases show LOH and mutation at the *TP53* locus, whereas the initial stages harbor the wild-type *TP53* gene (Pavelic et al., 2006). Immunohistochemical investigations of thyroid tumor samples have indicated the accumulation of both wild-type and mutant p53. p53 accumulation is observed not only in anaplastic and poorly differentiated forms where p53 mutation has been widely noticed, but also in well differentiated tumors in the absence of any p53 mutation (Soares et al., 1994). In both instances, the function of the accumulated protein is compromised. One of the accepted mechanisms of p53 inactivation is the altered interaction with Mdm2 used in the degradation of the p53 (see section 1.1 above). In the presence of the wild-type p53 protein, other mechanisms for inactivating p53 in thyroid cancers include cytoplasmic retention of the protein and over expression of *mdm2*. The enhanced expression of the *mdm2* gene is correlated with poor clinical outcome (Horie et al., 2001). Mutations in different locations of p53 appear to have different biological consequences that may lead to the development and progression of tumors through a range of mechanisms. In other cancers, the vast majority of the mutants create a p53 protein with an altered amino acid in the DNA binding domain (Soussi et al., 2005). Mutation at codons 213 and 238 are common in malignant thyroid cancers, while anaplastic carcinomas show most of the *TP53* gene mutations occurring at codons 238, 248 and 273 (Fagin et al., 1993). The patients carrying those mutations show lower survival rates, as well as lower rates of apoptosis in isolated tumor cells (Pavelic et al., 2006). Any of these changes in the *TP53* gene could affect the function of the p53 protein in thyroid cancer cells.

### 1.3 p53 functions as a transcription factor

Sequence specific transactivation of genes is an essential function of p53 as a tumor suppressor. The genes for several members of the cell cycle and cell death regulatory pathways are under the control of p53 in cells causing either their activation or repression of expression. The regulation of these genes is highly variable among different cancer cell lines (Yu et al., 1999). Literature suggests that aberrant regulation of target genes by p53 may lead to oncogenic activation or suppression of the expression of important genes leading to an imbalance in cell division or cell death and ultimately cancer (Vogelstein et al., 2000; Vousden & Lu, 2002). The p53 protein is known to regulate more than 50 different genes. A small subset of those genes in pathways regulating the cell cycle and apoptosis were studied here and are listed in Table 1.

| Gene containing p53 regulatory sequence | Pathway involved | Gene up-regulated or down regulated by wild-type p53 | Sequence of top strand used for DNA binding analysis (5′→3′) |
|---|---|---|---|
| ***p21-5′ site*** | Cell cycle | Up | CGAGGAACATGTCCCAACATGTTGCTCGAG |
| ***bax*** | Apoptosis | Up | GGGCTCACAAGTTAGAGACAAGCCTGGGCG |
| ***noxa*** | Apoptosis | Up | ATCTGAGGCTTGCCCCGGCAAGTTGCGCTC |
| ***survivin*** | Apoptosis | Down | AAGAGGGCGTGCGCTCCCGACATGCCCCGCG |
| ***cdc25c*** | Cell cycle | Down | GGGCAAGTCTTACCATTTCCAGAGCAAGCAC |

Table 1. The p53 genes and their regulatory sequences used for this study. Information presented here is from the IARC web site: http://www-p53.iarc.fr/index.html and Lacroix et al., 2006. One strand of the biotinylated double-stranded DNA sequences used for the binding studies is given.

For this study, we focused on the regulatory regions of 5 genes; 3 regulating apoptosis and 2 regulating the cell cycle (Table 1). **p21** is an important cell cycle regulatory protein which inhibits a wide variety of cyclin/cdk complexes essential for the transition between the phases of the cell cycle (Xiong et al., 1993). Expression of the *p21* gene is induced in a p53- dependent manner in response to cell stress, such as radiation (Dulic et al., 1994) or drug treatment (i.e. 5-fluorouracil) (Hernandez-Vargas et al., 2006). It has also been found that *p21* is activated in a p53-independent manner (Parker et al., 1995). **Bax** is one of the major members of the apoptotic pathway of cells whose gene is upregulated by wild-type p53 (Milhara et al., 2003). Bax is a pro-apoptotic member of the Bcl2 family, which functions on the mitochondrial membrane to promote cytochrome c release into the cell cytoplasm and subsequent events of apoptosis (Lindsten et al., 2000). Wild–type p53 has been shown to down-regulate ***survivin*** in cancer cells in response to chemotherapeutic agents (Hoffman et al., 2002; Mirza et al., 2002). In turn, this results in depletion of cells in the G2/M phase and induction of apoptosis (Zhou et al., 2002). Similarly ***cdc25C*** is an important gene associated with cell cycle regulation and its gene product belongs to the family of protein phosphatases that activates the cdk's by dephosphorylating a specific serine that promotes the entry of cells into the mitotic phase. In a normal cell, Cdc25C suppresses the inhibitory effect exerted by p53 on mitotic entry of cells. It has been shown

that the tumor suppressor p53 down-regulates the expression of the *cdc25A* gene, preventing abnormal cell proliferation (Nilson & Hoffman, 2000). The **Noxa** protein is an important mediator of apoptotic response induced by p53. This candidate encodes for BH3-only protein and is a pro-apoptotic member of the Bcl2 family, needed to initiate apoptosis in response to DNA damage (Huntington et al., 2009). As DNA binding is an essential component of transcriptional activation of a gene and the contribution of altered DNA binding by mutant p53 to its target gene sequences has not been fully elucidated, our present analysis of this function of p53 should provide insight into the expected regulation of gene expression.

### 1.4 Methods of studying protein-DNA binding

DNA binding by the p53 protein is known to regulate the expression of genes. We and others hypothesize that altered protein structure of mutant p53 might result in differential p53-DNA binding which could translate into abnormal target gene expression. The study of the DNA–protein interaction is done using a number of methods and each has its own advantages and disadvantages. The most common among them is the **Electrophoretic Mobility Shift Assay** (EMSA). In this assay, there is a shift in the migration of a DNA band on a gel in the presence of a DNA binding protein. Park and his colleagues (1994) demonstrated that the p53 mutant with changes at codon 273 (like the anaplastic thyroid cancer cell line ARO) has the ability to bind to the p53 consensus DNA sequence and activate transcription, whereas all the other mutants (at positions 156, 175, 223, 248 and 280) bound to the DNA, but did not transactivate the reporter gene. Their research also found that the p53 from the follicular thyroid cancer cell line WRO with mutation at codon 223 was able to bind to DNA, but was unable to activate transcription. It was concluded that cancer cells with mutation at codon 273 of p53 are very different from cells with mutations at other sites in the protein. One of the challenges faced with EMSA is that it does not easily provide quantitative values for the binding of protein to the DNA sequence. A study by Namba and colleagues (1995) utilized EMSA to show that wild-type p53 in nuclear extracts from cells exposed to radiation bound to *p21* and *gadd45* regulatory sequences and induced $G_1$ arrest through the accumulation of the p21 protein. **Scintillation Proximity Assay** (SPA) uses a radioactively labeled DNA sequence of interest that binds to p53 immobilized on scintillant containing beads and generates light which can be quantified. The intensity of binding is reflected as a higher number of counts (Gal et al., 2006; Chandrachud & Gal, 2009). The authors here have developed the SPA method to quantitate p53 DNA binding from baculovirus-expressed p53, as well as in extracts from human cancer cell lines. It has been shown that wild-type p53 from MCF-7 breast cancer cells treated with hydrogen peroxide and WRO (a thyroid cancer cell line with mutant p53) show greater affinity for the *cyclin G* gene regulatory sequence when compared to extracts from other cancer cell lines. **Fluorescence anisotropy** has also been utilized to study sequence specific DNA binding to several of the p53 target gene sequences by p53 protein obtained from bacterial expression (Weinberg et al., 2004, 2005). Fluorescence anisotropy measures the presence of the DNA binding protein as it alters the mobility of the fluorescently labeled DNA and as such, affects the polarity of the emitted light by the fluorophor. Heterologously expressed p53 protein forms (wild-type and truncated) have been shown to bind to gene regulatory sequences with differential affinities, particularly the genes within the apoptosis pathway. The **Streptavidin Magnetic Bead Assay** (SA) utilizes biotinylated DNA sequences and protein extracts

containing wild-type or mutant p53. This assay requires the use of streptavidin magnetic beads to separate the p53 DNA complex with the help of a magnet. DNA binding with p53 protein from tissue lysates of frozen breast cancer specimens has been carried out using streptavidin and biotinylated DNA. The results showed that some of the extracts demonstrated binding to the consensus sequence, whereas others failed to show such binding (Liu et al., 2001). Studies by Chandrachud and Gal (2009) demonstrated variable binding of wild-type and mutant p53 from MCF-7 and thyroid cancer cells, respectively, to different gene regulatory sequence by SA.

In order to better understand the role of p53 in thyroid cancer, we have analyzed the sub-cellular localization, post-translational modifications and the DNA binding specificity of the p53 from 3 cell lines; 2 thyroid cancer cell lines, ARO and WRO, both with mutant p53 and a breast cancer cell line, MCF-7, carrying wild-type p53 protein. We also studied the effects of oxidative stress (in the form of $H_2O_2$ treatment) on p53 level, localization and DNA binding activity in all 3 cell lines.

## 2. Materials and methods

### 2.1 Cell culture and hydrogen peroxide treatment

Thyroid cancer cell lines ARO and WRO (originally provided by Frances Carr Professor of Pharmacology, University of Vermont) were grown in RPMI-1640 (ATCC, Manassas, VA) supplemented with 10% fetal bovine serum (Thermofisher Scientific, Rockford, IL), 100U/ml penicillin and 100µg/ml streptomycin (Lonza, Walkersville,MD) at 37° C and 5% $CO_2$. MCF-7 cells (American Type Culture Collection (ATCC), Manassas, VA) were cultured following the same conditions as mentioned above except that they were grown in Minimum Essential Medium (ATCC). At about 80-85% confluence, the cells were treated with 0.2mM freshly prepared hydrogen peroxide ($H_2O_2$) (J. T Baker Inc., Phillipsburg, NJ) for three hours.

### 2.2 Cytoplasmic and nuclear extract preparation

Cells (either untreated or treated with $H_2O_2$) were harvested for their cytoplasmic and nuclear fractions using a published protocol (Jagelskà et al., 2002). Total protein concentrations (µg/µl) in the fractions were determined by bincinchoninic acid assay (BCA) (Sigma Chemical, St. Louis, MO) using bovine serum albumin (BSA) as a standard. The p53 (pg/µl) in the two fractions from the cell lines was estimated utilizing the Pantropic p53 ELISA Kit obtained from Calbiochem-EMD (La Jolla, CA).

### 2.3 Western blots

Following separation of proteins on 10% polyacrylamide gels (BioRad, Hercules, CA) and transfer to nitrocellulose membranes using the iBlot system (Invitrogen, Carlsbad, CA), p53 in the nuclear as well as cytoplasmic extracts was visualized with anti-p53 DO-7 (1:2000 dilution) (Calbiochem-EMD) by incubating the antibody with the nitrocellulose membrane in 5% BSA blocking solution in 1x Tris buffered saline for 3 hours. Actin in cytoplasmic extracts was visualized with anti-β-actin (mouse) (1:1000 dilution) (Sigma Chemicals) by incubating the membrane with antibody for 3 hours. The membranes were then incubated

with goat anti-mouse IgG-alkaline phosphatase (AP) (1:1000) secondary antibody (Southern Biotech, Birmingham, AL) in 5% BSA blocking solution in 1x Tris buffered saline for 1 hour and visualized with the colorimetric alkaline phosphatase substrate (KPL, Inc., Gaithersburg, MD) with an exposure time of 3-4 minutes. For p53 phosphoserine detection, p53 pSer15 was visualized with Phosphodetect™ anti-p53 ($pSer^{15}$) (Ab-3) (1:1000 dilution) (Calbiochem-EMD) by incubating the antibody with the membrane for 3 hours. p53 pSer392 was visualized with Phosphodetect™ anti-p53 ($pSer^{392}$) (Ab-4) (1:1000 dilution) (Calbiochem-EMD) by incubating the antibody with the membrane for 18-24 hours. The membrane was then incubated with donkey anti-rabbit IgG(H+L)-AP (1:1000) secondary antibody (Southern Biotech) in 5% BSA blocking solution in 1x Tris buffered saline for 1 hour and visualized with the colorimetric alkaline phosphatase substrate with an exposure time of 10-12 minutes. Signal intensities were observed by means of densitometry and analyzed using Quantity One software by BioRad.

### 2.4 Streptavidin magnetic bead assay (SA)

This assay based on a published protocol (Chandrachud & Gal 2009) was performed by incubating 50pg of p53 from ARO WRO, and MCF-7 nuclear and cytoplasmic extracts with 20pmoles of biotinylated DNA (30bp) (see Table 1 for sequences) in the other components used previously. Both the 'bound' and 'unbound' fractions along with a pre-bound fraction were separated on 10% SDS-PAGE gels (Bio-Rad), transferred to a PVDF membrane (Millipore, Bedford, MA) and probed for p53 with DO-7 as for the western blots (above) except that PVDF membranes were used and the secondary antibody was conjugated with horse radish peroxidase (HRP) (1:10,000) (Cell Signaling, Danvers, MA). The signal was visualized using a chemiluminescent substrate (Thermofisher Scientific). Signal intensities were quantified by means of densitometry and analyzed using the Quantity One software (Bio-Rad). The percentage of p53 bound to a particular gene regulatory sequence was calculated by adding the intensities in both the 'bound' and 'unbound' fractions and subsequently calculating the percentage in each fraction.

## 3. Results

We focused our studies on determining the functional status of p53 as a transcription factor and the potential role of phosphorylation as a p53 regulatory mechanism in untreated and $H_2O_2$ treated ARO and WRO thyroid cancer cells. To accomplish this, we determined the level of p53, its localization in the cell and phosphorylation and the p53 DNA binding specificity for target gene promoter sequences. The thyroid cancer cell lines ARO and WRO have mutations in the p53 within the DNA binding domain at positions 273 (R to H) and 223 (P to L), respectively (Fagin et al., 1993). In some cases, we used the breast cancer cell line, MCF-7 as a comparison for these thyroid cancer cells as it has wild-type p53 (Okumura et al., 2002).

### 3.1 p53 is predominantly located in the nucleus of ARO and WRO thyroid cancer cells

The level of p53 in nuclear and cytoplasmic fractions from untreated and $H_2O_2$ treated ARO and WRO thyroid cancer cells was determined using ELISA and compared to the total protein (Table 2). The level of p53 in nuclear and cytoplasmic fractions from untreated ARO and WRO thyroid cancer cells was also determined by western blot

analysis (Figure 1). p53 is detected predominantly in the nuclear fraction of all cell extracts with only a minor amount in the ARO cytoplasmic extracts. This latter localization may be an artefact of the extraction procedure or may indicate some retention of p53 in the cytoplasm. p53 was not detected in the cytoplasmic extracts from WRO cells (Figure 1). The relative amounts of p53 in ARO and WRO nuclear extracts are comparable for the same amount of total protein loaded (Figure 1). This observation is not consistent with the p53 quantitation by ELISA showing much lower levels of p53 in the WRO compared to the ARO cells (Table 2). The ELISA kit presumably uses different antibodies than what we used in the western blot analysis which may explain the discrepancy (the identity of the antibodies used in the ELISA kit was not provided by the company when requested).

### 3.2 Oxidative stress does not result in an increase or change the localization of p53 in ARO and WRO thyroid cancer cell lines

We have determined in our laboratory (Chandrachud & Gal, 2009) that $H_2O_2$ treatment of MCF-7 breast cancer cells (containing wild-type p53) induces the accumulation of p53 in the nuclear fraction of cell extracts. Using western blot analysis, we determined that the amount of p53 in ARO and WRO nuclear extracts does not change upon $H_2O_2$ treatment (Figure 2). This is corroborated by the data from the ELISA comparing the levels of p53 with total protein (Table 2). As expected, we observed an increase in the p53 level in MCF-7 breast cancer cells after treatment. $H_2O_2$ treatment also does not alter the localization of p53 in ARO or WRO thyroid cancer cells (Figure 1 and Table 2).

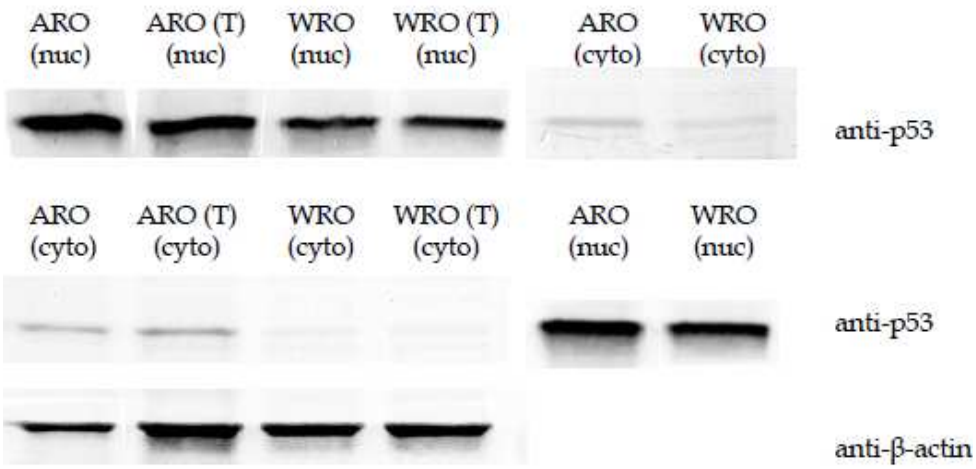



Fig. 1. p53 detection in nuclear and cytoplasmic extracts of ARO and WRO thyroid cancer cells. Western blot analysis shows the distribution of p53 in nuclear (nuc) versus cytoplasmic (cyto) fractions for both ARO and WRO untreated and $H_2O_2$ treated (T) cells. Total protein amount of 10μg was loaded in each well. β-actin was used as a loading control for cytoplasmic extracts. p53 is predominantly localized in the nuclear fractions from both cell lines.

| Extract | ARO | ARO(T) | WRO | WRO(T) | MCF-7 | MCF-7(T) |
|---|---|---|---|---|---|---|
| Nuclear p53 (pg/μl) | 203 | 211 | 43 | 36 | 8 | 18 |
| Cytoplasmic p53(pg/μl) | 31 | 32 | 9 | 18 | n/a | n/a |
| Nuc total protein (μg/μl) | 7.4 | 7 | 8.6 | 9.2 | 9 | 8 |
| Cyto total protein(μg/μl) | 8 | 4.3 | 4.7 | 4.4 | 6 | 4 |
| Ratio of nuclear p53/total protein | 27.3 | 29.4 | 5 | 4 | 1 | 2.3 |

Table 2. Level of p53 and total protein in the nuclear and cytoplasmic extracts of the cell lines. Nuclear and cytoplasmic extracts derived from untreated and cells treated with $H_2O_2$ for 3 hours (T) were analyzed for p53 by ELISA and for total protein by BCA as described in the Methods section. Two of the cytoplasmic extracts were not analyzed (n/a) for the p53 levels. The level of p53 is higher in the nuclear extracts than in the cytoplasmic extracts and higher in the thyroid cancer cell lines ARO and WRO compared to that in the breast cancer cell line MCF-7 with wild-type p53. Treatment does not increase the level of p53 in the thyroid cancer cells, but does cause an increase in MCF-7 cells as previously published (Chandrachud & Gal, 2009).

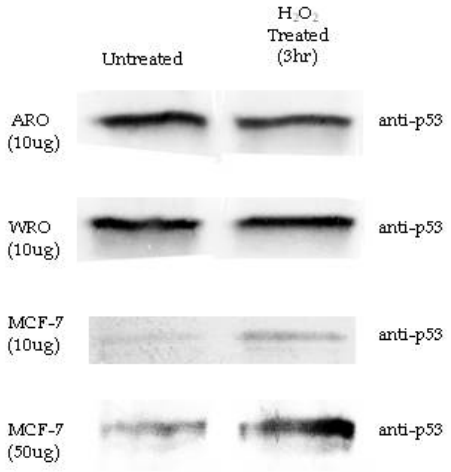
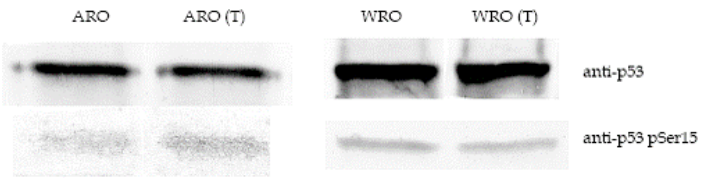



Fig. 2. Levels of p53 in nuclear extracts following $H_2O_2$ treatment. Western blots were used to monitor the level of p53 in nuclear extracts of ARO, WRO and MCF-7 cells initially (untreated) and following a 3 hour treatment with $H_2O_2$. Total protein amount of 10μg or 50μg was loaded into each well. The level of p53 does not change in ARO and WRO mutant thyroid cancer cells upon treatment, while the amount of p53 increases in MCF-7 cells after $H_2O_2$ treatment.

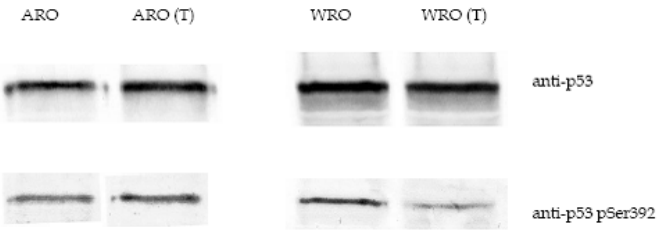
### 3.3 Phosphorylation of p53 is observed at S15 and S392 in thyroid cancer cells

Phosphorylation of specific residues on p53 has been linked to alterations in the functional status of p53 as a transcription factor (Xu, 2003). p53 is phosphorylated at residues 15 and 392 in extracts from ARO and WRO cells (Figure 3). Phosphorylation of p53 at S15 and S392 is not induced by oxidative stress. Interestingly, $H_2O_2$ treatment appears to decrease

phosphorylation of p53 at residue 392 in ARO and WRO treated cells (Figure 3B). Phosphorylation of p53 at residues 20 and 46 was not detected in the nuclear extracts from the thyroid cancer cells (data not shown).




A.




B.

Fig. 3. Western blot analysis of the phosphorylation state of p53 at residues 15 and 392. Nuclear extracts from ARO and WRO thyroid cancer cells containing 300pg of p53 (based on ELISA) were loaded on the gel, and the derived blot probed with antibodies to phosphorylated p53 at S15 (panel A) or at S392 (Panel B) and for total p53 (anti-p53). Phosphorylation of p53 at S15 does not appear to change in response to $H_2O_2$ treatment, while that modification at S392 appears to decrease after treatment (for WRO cells only).

### 3.4 DNA binding to some of the sequences by the p53 protein is detected

One measure of the functional status of p53 is the DNA binding and while some work has been done using the DNA consensus sequence with the p53 from these cell lines, we wished to look for differences in binding specificity to several of the more than 100 target sequences recognized by p53. Previously, we have shown that the p53 from these cell lines has somewhat different specificity for the regulatory regions from *p21*, *mdm2* and *cyclin G* (Chandrachud & Gal, 2009). We wanted to extend the sequences analyzed by including 4 other genes, 2 genes regulating the cell cycle and 2 belonging to the apoptotic pathway. Minimal binding was seen to the *cdc25C*, *bax* and *noxa* regulatory regions by the ARO and WRO nuclear extracts while DNA binding was detected with *p21* and *survivin* gene regulatory regions (Figure 4, Table 3, data not shown). ARO and WRO both bind similarly to *p21* and *survivin* gene regulatory sequences. In a previous publication, the p53 from nuclear extracts from ARO and WRO cells also bound to the *mdm2* and *cyclin G* regulatory gene sequences, both at 10-15% of the p53 bound for ARO and at about 5% bound for the p53 from the WRO cell nuclear extracts using SA (Chandrachud & Gal, 2009). DNA binding

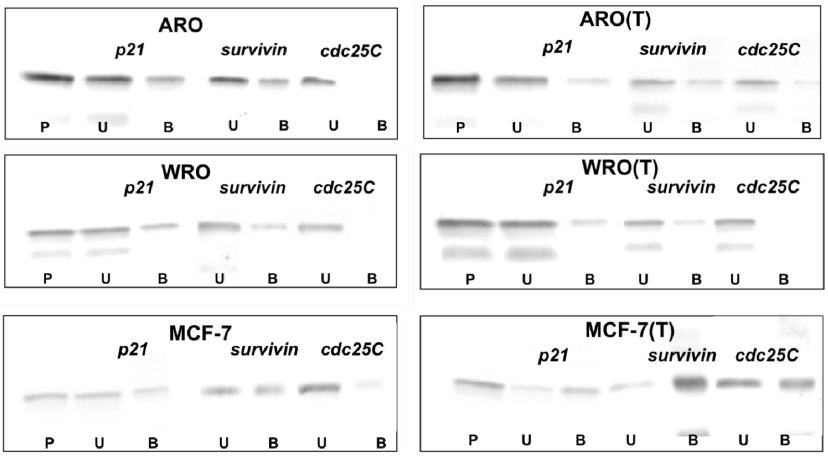



Fig. 4. DNA binding by p53 from nuclear extracts to various DNA sequences. DNA binding by 50pg of p53 to 20pmole biotinylated DNA sequences from the *p21*, *survivin*, and *cdc25C* promoter regions were performed. The p53 in the DNA bound (B) or unbound (U) fractions and an equivalent aliquot of the protein prior to binding (P) were detected using western blots. Nuclear extracts were derived from ARO, WRO and MCF-7 cells, untreated and following $H_2O_2$ treatment (T). DNA binding occurs to the *p21* and *survivin* gene promoters while no binding is detected to the *cdc25C* gene by the p53 from the ARO and WRO cells. Binding is detected to all of the sequences by the p53 from the MCF-7 cells, which increases upon treatment with $H_2O_2$.

| Gene | ARO | ARO(T) | WRO | WRO(T) | MCF-7 | MCF-7(T) |
|---|---|---|---|---|---|---|
| *p21* | 33 | 16 | 32 | 25 | 34 | 83 |
| *cdc25C* | 5.8 | 5 | 1.8 | 2 | 13 | 44 |
| *bax* | 1 | 1.8 | 0.5 | 2 | 11 | 45.6 |
| *noxa* | 1.5 | 0.9 | 0 | 0 | 14 | 56 |
| *survivin* | 33 | 15.6 | 32 | 28 | 47 | 76 |

Table 3. DNA binding specificity of p53 from the nuclear extracts from different cell lines. Nuclear extracts from the different cells untreated or treated with $H_2O_2$ for 3 hours (T) were prepared and used for the streptavidin DNA binding assay as described for each of the 5 sequences given. The numbers indicate the percent of total p53 in the extract that was bound to the particular sequence using western blots like those shown in Figure 4 as well as data not shown. The experiments using extracts from MCF-7 cells have been repeated at least 3 times, while those with ARO and WRO cell extracts have been repeated twice with the average difference between the replicate values being 1-6%. Binding was detected with the p53 from the ARO, WRO nuclear extracts to the *p21* and *survivin* regulatory regions only and decreased upon treatment with $H_2O_2$. Binding was detected to all 5 sequences by the p53 from the MCF-7 cells and increased 2-4X upon treatment with $H_2O_2$.

was also tested with the p53 in the cytoplasmic extracts, but no binding was detected in any of these extracts with the *p21* sequence used here (data not shown). Interestingly, we detected a slightly faster migrating p53 species in some of the extracts, particularly in the

cytoplasm of the thyroid cancer cells. This band is not present in the bound fractions using any of the biotinylated gene sequences, consistent with this protein representing a non-DNA binding form of p53. Although the presence of this lower band may be an artefact of the extraction procedure, there are several alternative forms of p53 known (Khoury & Bourdon, 2010). The identity of this faster migrating p53 protein is not known.

### 3.5 Changes in DNA binding following $H_2O_2$ treatment

In the previous publication, only the MCF-7 cells had been treated with $H_2O_2$ to activate p53 (Chandrachud & Gal, 2009). Here, that stress treatment was also applied to the ARO and WRO cells and the nuclear extracts were analyzed for alterations in DNA binding specificity (Figure 4 and Table 3). Insignificant binding is still seen to the *cdc25C*, *bax* and *noxa* regulatory regions, but for both thyroid cancer cell lines, treatment with $H_2O_2$ appeared to reduce the level of binding to the *p21* and *survivin* regulatory regions (from the ARO extract by about 50% while that from WRO by between 15 and 25%). In contrast, the binding by the wild-type p53 from the MCF-7 breast cancer cell line was detected for all 5 sequences, and binding to all 5 was enhanced between 2 and 4x after $H_2O_2$ treatment (Figure 4 and Table 3). This is consistent with previous work showing an increase in binding to the *cyclin G*, *mdm2* and *p21* regulatory sequences by the p53 from the MCF-7 cells (Chandrachud & Gal, 2009). Thus, there is a different DNA binding specificity of the p53 in MCF-7 cells once the cells are treated with $H_2O_2$, which is again different from that detected with the mutant p53 from the ARO and WRO cells.

## 4. Discussion

The functionality of p53 is essential for cells to maintain normal control of the cell cycle and apoptosis. We have looked at the characteristics of p53 from several angles in this chapter and all have provided useful information to better understand this tumor suppressor in the thyroid cancer cell lines, ARO and WRO. As noted above, the p53 in these cells contains a mutation resulting in a different protein from the wild-type protein present in the breast cancer cell line, MCF-7 that we have used for comparison in this work. We have looked at the level of the p53 protein and detected much higher amounts in the thyroid cancer cells compared to the level in the breast cancer cells. We have noted that the protein in ARO and WRO cells is primarily in the nucleus and that it shows phosphorylation at 2 serine residues (15 and 392). The DNA binding by p53 is similar in ARO and WRO cells but shows some significant differences compared to that detected in the MCF-7 cells. We have also compared each of those properties in cells that have been treated to oxidative stress and found that little changes in the p53 from the thyroid cancer cell lines while there is a significant change in the protein from MCF-7 cells. Each of these aspects will be examined further below.

The level of p53 protein observed by ELISA (Table 2) demonstrates a significantly higher level of the p53 present in the thyroid cancer cell lines versus the breast cancer cell line with ARO cell extracts having the most. Western blot analysis of the same extracts loaded at a 10μg total protein (Figure 2) shows a similar level of p53 in both untreated and $H_2O_2$ treated ARO and WRO cells which is higher than the level of protein observed in MCF-7 cells

loaded at both 10μg and 50μg of total protein. We are uncertain of the specificity of the p53 antibody provided in the Pantropic p53 ELISA kit, therefore it is difficult to speculate on the cause of the discrepancy between the ELISA and western blot results observed for the ARO and WRO cell lines (Table 2, Figure 2, respectively). It is evident by densitometry analysis (data not shown) and visual observation of the western blots that both ARO and WRO cell extracts contain very similar levels of the p53 protein. There are several mechanisms to explain the increased level of the p53 observed in the thyroid cancer cells. First, p53 is a transcription factor that is responsible for activating a subset of genes in response to DNA damage, hypoxia and genotoxic agents, such as etoposide and doxorubicin (Vassilev et al., 2004). Both thyroid cancer cell lines express mutations of the p53 protein in the DNA binding domain (ARO, R273H and WRO, P223L) potentially affecting its ability to bind DNA and potentially to induce *mdm2*, which is the primary regulator of p53 (Alarcon-Vargas & Ronai, 2002). Mdm2 binds wild-type p53 with high affinity and acts as a negative modulator of p53 transcriptional activity (Vassilev et. al., 2004). Therefore, the lack of induction of *mdm2* by the mutant p53 may be playing a direct role in the increased stability of p53, resulting in the higher levels of this protein observed in thyroid cancer cells. Another alternative is that the mutant form of p53 is unable to interact with Mdm2 and thus is not targeted for degradation (Prives & White, 2008). Since we do not know the status of Mdm2 (null, wild-type or mutant) in ARO and WRO cells, we can not say definitively whether or not lack of *mdm2* transcription is the causative factor. Research in other cell systems indicates that it is a plausible root cause and could be explored in the future with these thyroid cancer cell lines.

The p53 protein observed in ARO and WRO cells is phosphorylated at serine residues 15 (S15) and 392 (S392) (Figure 3). This is significant because both of these post-translational modifications are known to play important roles in the regulation by Mdm2 by either disrupting interactions between Mdm2 and the N-terminal transactivation domain of p53 (S15) (Xu, 2003) or by blocking ubiquitin-dependent degradation and nuclear export of p53 via the modification in the C-terminal regulatory domain (S392) (Kim et al., 2004). Studies conducted by Kim and colleagues (2004) demonstrate that modification of S392 blocks the binding of the human papilloma virus E6 protein to the p53 and promotes nuclear localization of p53, both of which confer p53 protein stability. We demonstrate that p53 accumulates and is largely retained in the nuclear compartment of thyroid cancer cells in both untreated and $H_2O_2$ treated cells (Figures 1 and 2). Since the p53 nuclear localization signal (NLS) (residues 316-325) and nuclear export signal (NES) (residues 356-362) are in proximity to the oligomerization domain, Liang and Clarke (2001) have proposed that tetramerization of p53 can inhibit nuclear export by masking the NES and /or NLS required for transport. The observation that the p53 in ARO and WRO thyroid cancer cells is retained in the nucleus and phosphorylated at S15 and S392 indicates that the mutant p53 is likely tetrameric and potentially functional in the cells, although not necessarily able to regulate transcription.

The WRO cells, derived from a follicular carcinoma, are heterozygous for the P223L mutation (Liu et al., 2008). One way in which p53 is regulated in tumor cells is through trans-dominant suppression of wild-type p53 function by a mutant protein. Simultaneous expression of wild-type and mutant p53 has been known to result in hetero-tetramerization

of two types of monomers, which could result in a fully intact protein with loss of wild-type protein activity (Chan et al., 2004; Srivastava et al., 1993; Unger et al., 1993). The ARO cells carry the mutation R273H (one of the most common sites altered in all cancers); however, we do not have confirmation as to whether these cells contain a wild-type p53 allele or are homozygous for the mutation. The likelihood for the oligomerization of mixed tetramers would also explain the accumulation of a fully intact, and thus potentially functional protein retained in the nucleus as we demonstrated in this paper.

A variety of treatments are known to stabilize p53 by blocking the Mdm2-mediated degradation of the protein. In this study, we focused on oxidative stress and it's effect on the p53 functionality in the thyroid cancer cell lines as we and others have seen changes in this protein following $H_2O_2$ treatment of the breast cancer cell line, MCF-7 (Chandrachud & Gal, 2009; Chuang et al., 2002). We did not see significant changes in the level or localization of the mutant p53 protein following $H_2O_2$ treatment of ARO and WRO cells. This protein is present at a much higher level in these thyroid cancer cells when compared to the level in the MCF-7 cells. It is possible that the protein is already at maximum level and thus can not be further increased. As noted above, the accumulation of p53 could be due to the lack of interaction of the protein with Mdm2 even before oxidative stress treatments, so there may be no change upon treatment. It is also possible there is an alteration in the transducer of the oxidative stress signal (such as the 2-Cys PRX Tpx1 protein) (Veal et al., 2007) in the ARO and WRO cells such that no signal is actually transmitted to cause a change in the accumulation of the p53 protein. One of the upstream regulators of p53 that has been implicated in regulation of p53 activation and stabilization in response to DNA damage is Chk2 (Shieh et al., 2000). Chk2 has been found to induce phosphorylation on Ser 20 of p53 in response to stress which leads to interruption in p53-Mdm2 interaction and a subsequent decrease in p53 ubiquitination (Hirao et al., 2000). We did not observe any phosphorylation at Ser 20 of the p53 from any of the cell lines studied. Phosphorylation of specific serine residues on the p53 protein has been shown following different stress treatments (Hernandez-Vargas et al., 2008). The p53 from ARO and WRO cells has been demonstrated here to be phosphorylated at S15 and S392 in untreated cells (Figure 3). $H_2O_2$ treatment apparently reduces the level of phosphorylated S392 in WRO cells with no change in the level of this modification in ARO cells. Whether those modifications affect the DNA binding activity of p53 is currently under investigation.

The p53 transcription factor regulates genes in the cell cycle and apoptosis pathways. We monitored DNA binding to 5 genes from these 2 pathways and noted all 5 were recognized by the wild-type p53 from the MCF-7 cells. However, only 2 sequences were recognized by the mutant p53 proteins, those derived from the *survivin* gene which blocks apoptosis and from the *p21* gene regulating the cell cycle. Previous studies link survivin to dedifferentiation in thyroid cancer (Ito et al., 2003), and over-expression of *survivin* has been reported in papillary thyroid carcinomas (Antonaci et al., 2008). Also, it has been reported that increased *survivin* expression is found in the initial stages of colorectal cancer tumor development (Kawasaki et al., 2001). Wild-type p53 binds to the *survivin* regulatory sequence and down-regulates the gene to reduce survival in response to apoptotic stimuli. As mutant p53 also binds to the regulatory element of *survivin*, this gene may be repressed in ARO and WRO cells. The p21 protein blocks cell cycle progression, and its gene is

upregulated by wild-type p53 in the event of cellular stress. The mutant p53 proteins from ARO and WRO cells bind the *p21* regulatory element as well, suggesting the p21 protein may also be present in these cells. It is known that some p53 mutants cause growth arrest through upregulation of *p21* (Ludwig et al., 1996). Alternatively, some p53 mutants may have mechanisms of transcriptional activation different from the wild-type protein. In fact, previous work with extracts from WRO cells indicated that the mutant p53 could bind to a DNA sequence, but could not upregulate the gene (Park et al., 1994). It is possible that the binding to the DNA sequences regulating the *p21* and *survivin* genes by mutant p53 may actually prevent binding by wild-type p53 or other transcription factors and in that way disrupt the normal regulation. We did note a modest reduction in the binding to these regulatory sequences by mutant p53 after oxidative stress, while the p53 from the MCF-7 cells had enhanced binding to these DNAs after treatment (Figure 4 and Table 3). This may support the possibility of negative regulation of these genes by the mutant p53. The level of mRNA for these two genes in these cell lines would have to be examined before and after $H_2O_2$ treatment to test this hypothesis. We observed that the p53 proteins from both ARO and WRO cells do not show any binding to the cell cycle regulatory gene *cdc25C* nor to the regulatory sequences of the pro-apoptotic genes *bax* and *noxa*. Thus, these tumor cells could survive by altering regulation of the cell cycle and the apoptotic pathways. Wild-type p53 down-regulates *cdc25C* to restrict entry into mitosis but upregulates *bax* and *noxa* to promote cell death. Thus, these genes would not be regulated by the mutant p53s resulting in less inhibition of entry into mitosis and a lack of promotion of cell death. The disruption of both pathways would result in growth promotion of the cancer cell lines. These data are in agreement with low apoptotic rates in isolated anaplastic carcinoma cells reported previously (Pavelic et al., 2006).

We have only studied the binding of the p53 proteins here to a small subset of the more than 100 regulatory sequences recognized by the wild-type protein (Menendez, et al., 2009; Riley et al., 2008). The DNA binding recognition of mutant p53 is specific to the sequence of the mutant protein. It is possible with future work in this direction to predict a consensus DNA binding sequence which is exclusively recognized by mutant p53 proteins and thus could explain altered DNA binding and transcriptional activation/ repression of mutant p53 target genes. In many previous publications, only the wild-type consensus DNA sequence has been used to study the p53 DNA binding in different cells and following different treatments. We are aware that DNA binding is not the full picture. For instance, the p53 from both the ARO and WRO cells bound the consensus sequence, but only the ARO cells transactivated the gene regulated by a promoter containing this sequence (Park et al., 1994). Mutant p53 may control transcription of target genes distinct from those regulated by the wild-type protein supporting the concept of a gain-of-function mutation in this master regulator (Deppert et al., 2000, Sigal & Rotter, 2000). Elucidating the molecular mechanisms behind gain-of-function by mutant p53 has proven to be difficult since the observed oncogenic effect often varies with the system under study.

## 5. Conclusion

The p53 in the ARO and WRO thyroid cancer cells is likely tetrameric as the protein is found in the nucleus and is phosphorylated. This would allow these proteins to affect transcription either directly or via interaction with the wild-type p53 protein. Oxidative stress does not

appear to alter the p53 from the thyroid cancer cells in the same way compared to the wild-type protein in breast cancer cells suggesting either altered sensing or already maximal over-produced protein. Our work showing differential DNA binding specificity of mutant and wild-type p53 supports the importance for continuing to study the binding to specific gene regions providing a fuller spectrum of information about the changes in functionality of the p53 protein in cancer cells. Future studies in this direction may identify mutant p53 regulated genes whose action promotes the gain-of-function attribute that makes the thyroid cancers carrying these mutant proteins much more aggressive.

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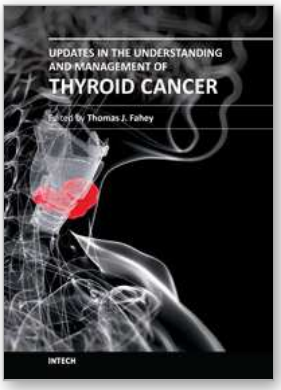


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