交 通 大 學 生 物 科 技 研 究 所

博 士 論 文

異常的 PDGFR-α 基因表現
對誘發甲狀腺濾泡癌癌化之探討

The significance of aberrant gene expression of PDGFR-α in the carcinogenesis of follicular thyroid carcinoma

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中文摘要:

甲狀腺腫瘤約佔所有腫瘤的 1%。腫瘤會發生在所有的年齡層，而且會隨著年齡的增加發生率也會增加。女性與男性罹患的比率約 3:1。目前，確定可行的診斷方式，是藉著組織學的觀察。但臨床上發現，有些早期的吸引性細胞學檢查，有時是不可信賴的，特別是針對濾泡型的甲狀腺腫瘤。通常濾泡型腫瘤或較少數的乳突型腫瘤，病理學家無法僅以吸引性細胞學檢查的結果，做出正確的診斷。

為了發展可以早期偵測的分子標的，我們首先藉著描繪出在不同的甲狀腺腫瘤組織與細胞株的表現具有差異的基因，從其中尋找具有潛力的腫瘤標的基因。cDNA 表現基因陣列(cDNA expression array)的技術，在本研究中用來描繪出在甲狀腺濾泡癌中，差別表現的基因，以顯現適合的腫瘤標誌。針對在濾泡型的甲狀腺腫瘤，表現具有差異的基因，進行進一步的研究，將有助於瞭解，這些基因改變在甲狀腺腫瘤癌化過程中，所扮演的角色。

在利用了 cDNA 表現基因陣列的技術分析差別表現基因過後，我們發現 PDGF-A 與 PDGFR-α 的 mRNA 相較於良性的結節性增生細胞(nodular hyperplasia cells)而言，在甲狀腺濾泡癌細胞株中，具有高度表現。因此，這些結果引起我們的動機，想瞭解是否具有 PDGF 相關的自分泌活化(autocrine activation)的現象，存在甲狀腺腫瘤細胞中且在癌化過程中，扮演重要角色。PDGFR 本身具有激脢活性(kinase activity)，當 PDGF 配位蛋白(ligand)結合上受體蛋白(receptor)時，可活化位於細胞內激脢活性。已知 PDGFR 活化與一些細胞的癌化，具有相關性，包含星狀細胞瘤(astrocytomas),寡樹突神經膠質瘤(oligodendrogliomas),及神經膠質胚胎細胞瘤(glioblastoma)。在 cDNA 表現基因陣列的分析，我們發現在濾泡癌細胞中，PDGF-A 與 PDGFR-α 之 mRNA，皆會增加表現。之後，利用西方墨點法分析，確認在濾泡癌細胞中，PDGF-A 與 PDGFR-α 蛋白，也會提升表現。更進一步，觀察 PDGFR-α 的磷酸化現象，發現利用針對第 720 位置的酪胺酸磷酸化專一性抗體，在濾泡癌細胞中，可偵測到磷酸化現象。而 PDGFR-α 所具有的酪胺酸激脢活性，可以被抑制劑 tyrphostin AG1295 所抑制，而且顯現隨著抑制劑劑量的增加，

而提升抑制腫瘤細胞增生的作用。另一方面，在免疫組織呈色實驗(immunohistochemistry)中，結果顯示 PDGFR-α 蛋白的表現，主要是呈現在甲狀腺濾泡周邊(thyroid follicle)且是與惡性的腫瘤階段(tumor stage)，呈現統計上的相關性。這些發現暗示，藉著 PDGFR-α 所進行的自分泌活化現象，在甲狀腺細胞的癌化過程中，扮演重要的角色。

進一步為了確認是否能藉著抑制這不正常的訊息傳遞，能夠達到解除癌化的可能性。我們檢驗 PDGFR-α siRNA 與 tyrphostin AG1295，這兩種方式，來觀察對抑制 CGTH W-1 細胞增生的功效。目前，已知有數種酪胺酸激脢抑制劑，可以阻擋具酪胺酸激脢活性的受體之訊息傳遞，從而抑制不正常的訊息傳遞。這裡我們設計小而具有干擾特性的 RNA (short interfering RNAs; siRNA)專門針對 PDGFR-α 基因，且達到壓制濾泡型的甲狀腺腫瘤細胞(CGTH W-1)增生的效果。即時定量聚合脢連鎖反應(real-time quantitative PCR)、流式細胞儀(flow cytometry)測定、免疫螢光細胞染色(immunofluorescence cell staining)及 MTT 測定後，結果顯示送入 PDGFR-α siRNA 的 CGTH W-1 細胞，可達到減少細胞中 PDGFR-α mRNA 的表現程度。之後，可以達到減少 CGTH W-1 細胞表面受體的分子數目及最後可以達到抑制細胞增生的效果。而利用 non-silencing siRNA 所進行的對照組實驗，則不管在 PDGFR-α 基因的表現或者是細胞增生，皆無顯著影響。

最後，我們比較利用 PDGFR-α siRNA 與 tyrphostin AG1295，在抑制 CGTH W-1 細胞增生的影響，發現利用 PDGFR-α siRNA 時，功效的呈現，相對於 tyrphostin AG1295，需要多 24 小時的時間，才能達到顯著的效果;但是它的作用可以達到 240 小時(第十天)之久。這些發現指示了 PDGFR-α siRNA 可以是一個有潛力的工具，來抑制不正常的 PDGFR-α 基因表現。另外也進一步的證明，在甲狀腺細胞癌化過程中，PDGFR-α 基因的表現，扮演重要的角色。

Abstract:

Thyroid cancer accounts for about 1% of all cancers. Thyroid cancer occurs at all ages and increases frequently in each decade. The female:male ratio is about 3:1. Definite diagnosis is possible only by histological examination. Fine needle aspiration cytology (FNAC) reports are sometime unreliable, especially for follicular thyroid tumors. Frequently, the pathologist cannot diagnose follicular carcinoma on FNAC or (to a lesser extent) papillary carcinoma.

To develop an early detection method by using biological markers, we have first searched the potent tumor markers by outline the differentially gene expressions in various thyroid tissues or thyroid cell lines. The cDNA expression array technology is utilized herein to profile differentially expressed genes from human follicular thyroid carcinoma and reveals new tumor markers. An expression profile of genes that are associated with malignant process of follicular thyroid cancer was further discussed. Further investigation is required to understand the precise relationship between the altered expression of these genes and the malignant process of follicular thyroid cancer.

After analysis of differentially gene expression by a cDNA microarray technique, we found that mRNA of PDGF-A and PDGFR-α were highly expressed in thyroid carcinomas but not in nodular hyperplasia cells. These results cause the motive to understand whether PDGF autocrine activation exists in thyroid cells and play a crucial role in carcinogenesis. Platelet-derived growth factor receptor (PDGFR) possesses a kinase activity and can be activated through binding with PDGF. The activation of PDGFR is associated with the carcinogenesis of some cell types, including astrocytomas, oligodendrogliomas, and glioblastoma. In a cDNA microarray analysis, we discovered the over-expressed mRNA of both PDGF-A and PDGF α-receptor in thyroid carcinoma cells. And the elevated protein expressions of PDGF-A and PDGF α-receptor in thyroid carcinoma cells were confirmed by a

western blot analysis. Furthermore, the phosphorylation of PDGF α-receptor measured by an antibody against Tyr 720-phosphate was found in thyroid carcinoma cells. The tyrosine kinase activity of PDGF α-receptor was inhibited by tyrphostin AG1295 and showed a dose-dependent inhibition for cell proliferation. In an immunohistochemistry study, data showed that the expression of PDGF α-receptor was primarily localized around the follicle and significantly correlated with malignant tumor stage. These findings imply that autocrine activation of PDGF-α receptor plays a crucial role in the carcinogenesis of thyroid cells.

To identify whether inhibition of the aberrant signal transduction could have the potential to dismiss the possibility of carcinogenesis, we examined the efficacy of PDGFR-α siRNA and tyrphostin AG1295 on repressing cell proliferation of follicular thyroid carcinoma cell line (CGTH W-1). Some tyrosine kinase inhibitors have shown to block the tyrosine-like receptors and achieve the inhibition of some aberrant signal transduction. Here we designed short interfering RNAs (siRNA) specific for PDGFR-α to repress cell proliferation in CGTH W-1. Real-time quantitative PCR, flow cytometry, immunofluorescence cell staining, and MTT assay results demonstrated that the transfected CGTH W-1 cells reduce the cellular PDGFR-α mRNA level, reduce the PDGF α-receptor in cell membrane, and repress cell proliferation. While control studies of non-silencing siRNA showed no significant effects in PDGFR-α expression and cell proliferation.

Finally, we compared the effects of repressing cell proliferation of CGTH W-1 cells by PDGFR-α siRNA and a tyrosine kinase inhibitor, tyrphostin AG1295. PDGFR-α siRNA required 24 hours more than tyrphostin AG1295 to show significant inhibition of cell proliferation, but the effects last up to 240 hours. The findings indicate that the PDGFR-α siRNA could be a potential tool to suppress aberrant PDGFR-α gene expression and furthermore show that PDGF-α receptor plays a crucial role in the carcinogenesis of thyroid cells.

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I. INTRODUCTION

The thyroid gland is the biggest gland in the neck and situated in the anterior neck below the skin and muscle layers. The thyroid gland takes the shape of a butterfly with the two wings being represented by the left and right thyroid lobes which wrap around the trachea. The function of the thyroid is to make thyroid hormone. This hormone has an effect on nearly all tissues of the body where it increases cellular activity. The function of the thyroid therefore is to regulate the body's metabolism.

1.1 Common Thyroid Disorders

The thyroid gland is prone to several very distinct disorders, some of which are extremely common. These disorders can be divided into four groups, including those concerning the production of hormone (too much, or too little), those due to increased growth of the thyroid causing compression of important neck structures or simply appearing as a mass in the neck, the formation of nodules or lumps within the thyroid which are worrisome for the presence of thyroid cancer, and those which are cancerous[1].

- Goiters: A thyroid goiter is a dramatic enlargement of the thyroid gland. Goiters are often removed because of cosmetic reasons or, more commonly, because they compress other vital structures of the neck including the trachea and the esophagus making breathing and swallowing difficult. Sometimes goiters will actually grow into the chest where they can cause trouble as well. X-rays will help explain all types of thyroid goiter problems.
- Hyperthyroidism: Hyperthyroidism means too much thyroid hormone. Current methods used for treating a hyperthyroid patient are radioactive iodine, anti-thyroid drugs, or surgery. Each method has advantages and disadvantages and is selected for individual patients. Many times the situation will suggest that all three methods are

appropriate, while other circumstances will dictate a single best therapeutic option. Surgery is the least treatment selected for hyperthyroidism.

- Hypothyroidism: Hypothyroidism means too little thyroid hormone and is a common problem. In fact, hypothyroidism is often present for a number of years before it is recognized and treated. Hypothyroidism can even be associated with pregnancy. Treatment for all types of hypothyroidism is usually straightforward.
- Thyroid Cancer: Thyroid cancer is a fairly common malignancy, however, the vast majority have excellent long term survival.

1.2 Incidence and distribution of thyroid cancer

The annual incidence of thyroid cancer varies considerably in different registries, ranging from 1.2-2.6 per 100,000 individuals in men and from 2.0-3.8 per 100,000 in women [2, 3]. It is particularly elevated in Iceland and Hawaii, being nearly two times higher than in North European countries, Canada and the USA. In Hawaii, the incidence rate of thyroid cancer in each ethnic group is higher than that registered in their country of origin [4], and it is particularly common among Chinese males and Filipino females. Most of the differences are probably due to ethnic or environmental factors (such as spontaneous background radiation) or dietary habits [5], but different standards of medical expertise and health care may also play a role in the efficiency of cancer detection. The American Cancer Society indicated incidence in the USA of nearly 10/100,000 population in 2003. The reported incidence has been increasing at more than 5% every year for a decade.

In sharp contrast with these data concerning the incidence of clinical thyroid cancer, is the prevalence found in autopsy series or screening programs. Autopsy studies indicate a surprising frequency ranging from 0.01 to over 2.0% [6, 7]. A survey of consecutive autopsies

found 2.7% of thyroids to harbor unsuspected thyroid cancer [7]. Another 2.7% had discrete benign adenomas, and nearly half showed nodularity. The high prevalence was attributed to careful examination of the gland, but probably also reflects a highly selected group of older patients dying in a hospital. Up to 6% of thyroid glands in autopsied adults in the United States, and over 20% in Japan, also harbor microscopically detectable foci of thyroid carcinoma, which are believed to be of no biologic significance. Altogether autopsy studies suggest that thyroid cancer is in many instances not diagnosed during life or is not the immediate cause of death. Both suggestions are in accord with the rather leisurely growth of the majority of thyroid tumors, especially the frequent papillary types. The annual mortality from thyroid cancer in 2003 was 5 per million for men and 6 per million for women [8]. The discrepancy between incidence and mortality reflects the good prognosis for most thyroid cancers. Recent statistics suggest about 6 deaths /million in the USA.

Thyroid tumors are rare in children and increase in frequency in each decade. The variety of tumor is also related to age. Carcinomas are three times as frequent in women as in men. In the past, it was generally believed that thyroid tumors were more frequent in areas of endemic goiter, and reports from Colombia and Austria support this association [9]. More recent studies suggest that in iodine deficient countries the number of nodules is increased and, as a consequence, also the number of thyroid cancers is increased [10]. Surveys conducted in the United States found no relation between usual geographic residence and incidence of thyroid cancer.

1.3 Pathology

Pathologists are agreed that there are peculiar difficulties in the classification and diagnosis of malignant tumors of the thyroid. The histologic changes required for diagnosis of carcinoma include absence of a true capsule, invasion of surrounding normal tissue, invasion

of blood and lymph channels, loss of normal follicular architectural arrangements, and cellular abnormalities such as an increase in the ratio of nucleus to cytoplasm, enlarged vesicular nuclei, nuclear folding, increased mitoses, and hyperchromasia of the nucleus. Obviously the presence of distant metastases is the most certain criterion. Most studies of the disease agree that the ordinary criteria of malignancy have little prognostic value in thyroid tumors, except perhaps in the wildly growing anaplastic tumors.

The general experience of pathologists has been that, in the absence of irradiation, the substrate in which thyroid tumor forms is usually like normal thyroid tissue or displays the changes of multinodular goiter or adenoma in approximately the proportion found in any sampling of the general population [11]. There is a slightly increased frequency of association with benign adenomas and with Hashimoto's thyroiditis [12]. Lymphomas are associated with Hashimoto's thyroiditis, and there is considerable evidence that lymphoma actually evolves from a gland with thyroiditis [13].

Multicentricity is a common feature of thyroid cancer, especially papillary cancer. Innumerable separate foci are sometimes found. Estimates of multicentricity range from 20 to 80% [14, 15]. Whether this phenomenon represents truly multicentric sites of origin or intrathyroidal dissemination is not clear. This multifocality is thought to be one cause of recurrences in patients treated by subtotal rather than total thyroidectomy.

Both papillary and follicular tumors may appear as small (less than 1.5-cm) tumors surrounded by a densely fibrotic reaction. Although it is frequently said that these "occult" (because they may be found incidentally at operation) tumors are benign, the original report by Hazard [16] and subsequent studies show that cervical lymph node metastases occur [17]. Occasionally pathologic examination suggests conversion of differentiated papillary or follicular cancers into anaplastic forms or conversion of an adenoma into a carcinoma.

1.4 Causes of Thyroid Carcinoma

Most, if not all, thyroid adenomas are monoclonal, as, presumably, are most carcinomas [18]. Colloid nodules may be either mono- or poly-clonal. Thus, tumors represent the persistent proliferation of the progeny of one cell which has somehow escaped the mechanisms which maintain normal cell division[19].

The process of carcinogenesis is thought to be a series of events induced by genetic and environmental factors which alter growth control. These factors may be considered as "initiators" and "promoters". Initiators include such agents as chemicals and irradiation which induce tumors, and promoters are agents such as phenobarbital, which in rats augments TSH secretion and radically increases tumor development. In man x-ray treatment is the sole known initiator, and other than elevated TSH, no promoters are known. Compounds such as phenobarbital, Dilantin and polychlorinated biphenyls (PCBs), which are known thyroid tumor promoters in animals through liver microsomal hormone degrading enzyme induction leading to increased thyroid hormone metabolism, do not appear to have a detectable adverse effect in man in doses usually employed [20].

1.5 Oncogenes in thyroid tumors

More than 30 "oncogenes" have been recognized in the human genome. These genes, normally silent, can become activated by chromosomal translocations, deletions, or mutations, and then can transform normal cells into a condition of uncontrolled poliferation. Most oncogenes appear to be closely related to normal growth factors, genes that control cell division, or to hormone receptors. In general, these genes, when turned on, promote cell growth and cell division and repress differentiation. Typically activation of one such gene

may not be enough to produce malignancy, but if accompanied by expression of another oncogene, or if gene mutation or reduplication occurs, the cell may progress toward a malignant potential. Information on expression of oncogenes in human thyroid tissue is rapidly accumulating. Expression of *c-myc* is stimulated in normal thyroid cells by TSH, and the proto-oncogene is expressed in adenomas and carcinomas. Activating mutations of *h-ras* at codons 12, 13, and 61, and over-expression of *h-ras*, are found in adenomas and carcinomas, but *h-ras* mutations are also found in nodular goiter tissue [21], suggesting that *h-ras* mutations could be an early event in carcinogenesis [21, 22]. Other studies, it should be noted, find *ras* mutations unusual [23].

Vecchio and co-workers [24] cloned an oncogene which is frequently and specifically expressed in papillary thyroid cancers. This oncogene is found on chromosome 10 (the area of the *MEN I* gene as well), and involves an intrachromosomal rearrangement of the tyrosine kinase domain of the *ret* oncogene so that it is attached to one of three different promoters, producing retPTC-1, retPTC-2, and retPTC-3. One of these translocation products is found in 20-70% of papillary cancers. This rearrangement leads to constitutive expression of the oncogene. It has been shown that intra-thyroidal expression of the ret/PTC1 oncogene can induce thyroid cancer [25]. *BRAF* mutations are also frequent in papillary carcinoma, and undifferentiated cancers that have arisen form papillary tumors[26].

Recently a mutational change has been associated with follicular thyroid cancers. In 5 of 8 follicular thyroid cancers, Kroll et al [27] found translocation of the DNA binding domain of PAX8 to domains A-F of the peroxisome proliferator-activater receptor (PPAR) gamma1 gene. The fusion oncogene is able to transform thyrocytes, so appears to be able to produce malignancies[28]. Although initially thought to be exclusively present in follicular cancers, it is now known to be also present in follicular adenomas [29]. Mutation or deletion of the *p53* tumor suppressor gene is found in some differentiated thyroid cancers, and many

undifferentiated cancers. This genetic deletion may be one of the final steps leading to anaplastic cancer growth (Fig.1). Simian virus 40-like sequences are found in many thyroid cancers, as well as other cancers, and the Tag gene sequence found is known to be oncogenic in animal models [30]. Mutated and non-functional thyroid hormone receptors are recognized in up to 90% of PTC, suggesting a role in oncogenesis, but other workers find these mutations to be rare. [31,32]. The tumor suppressor gene *TSG101* is over-expressed in most PTCs [33]. Overexpression of many other genes, including *galectin-3*, *Thymosin beta-10*, *hTERT*, *CD97*, *CD26*, *VEGF*, has been detected, but a question is whether these changes represent the result of carcinogenesis.

Mutations in the proteins involved in the TSH receptor-G protein-adenyl cyclase kinase signal transduction pathway also play a role in tumor formation. Activating TSH receptor mutations have been found by Vassart and co-workers [34] to be the cause of most hyperfunctional nodules, and are now known to be common in "hot" nodules in patients with multi-nodular goiter. These mutations involve the extracellular loops of the transmembrane domain and the transmembrane segments, and are proven to induce hyperfunction by transfection studies. However these mutations are not associated with cancer formation. Mutations in the stimulatory GTP binding protein subunit are also present in some patients with hyperfunctioning thyroid adenomas [35]. TSH-R mutations are, however, unusual in thyroid cancer [36], excepting hyperfunctional adenomas. TSH-R expression tends to be lost as cancers de-differentiate, and persistence of expression is associated with a better prognosis [37].

In addition to positive genetic factors, carcinogenesis frequently involves loss function of tumor suppressor genes. This has been proven in hereditary retinoblastoma. These genes are normally present on both sets (maternal and paternal) of chromosomes. In retinoblastoma the inherited lack of one suppressor (*RB*) gene does not cause disease, but if a genetic event

(deletion, recombination, mutation, etc.) causes failure of expression of the second allele, cancer derives. Deletion of the tumor suppressor genes, *p53* and the *RB* gene, have been found in differentiated and undifferentiated thyroid cancer [38]. Many chromosomal rearrangements are found in Hurthle cell tumors, and correlate with tumor recurrence [39].

1.6 Follicular thyroid carcinoma

Follicular thyroid carcinoma has a top incidence in the fifth decade of life in the United States and accounts for about one-quarter of all thyroid carcinomas [40-42]. In past decades, follicular carcinomas arrived up to 50% of thyroid malignancies in Europe. The high incidence may partly be explained by iodine deficiency, but, more likely, was due to histological miss-classification when the follicular variant of papillary thyroid carcinoma was not recognized as papillary but rather was classified as follicular. It is a slowly growing tumor and frequently is recognized as a nodule in the thyroid gland before metastases appear. Variation in the cellular pattern ranges from an almost normal-appearing structure to anaplastic tissue that forms no follicles or colloid. The insular variant of follicular tumor tends to be more aggressive [43]. The carcinoma is three times as common in women as in men. At operation one-half to two-thirds of these tumors are resected. Tumors that are small and well circumscribed tend to be less lethal than those actively infiltrating local structures. Local direct invasion of strap muscles and trachea is characteristic of the more aggressive tumors [44]. Resection depends on this feature, and death may be caused by local invasion and airway obstruction. The patients of minimally invasion have a better prognosis than those of highly invasion.

Follicular carcinomas tend to invade locally and metastasize distantly, rather than to local nodes, and are especially prone to metastasize to bone or lung. In a Massachusetts

General Hospital series [45], one-half of patients with metastases at the time have been diagnosed to possess metastasis. Bone metastases are usually osteolytic, rarely osteoblastic, and the alkaline phosphatase level is rarely elevated. The tumor and metastases often retain an ability to accumulate and keep iodide, and are therefore sometimes susceptible to treatment with radioactive iodine(RAI). Indeed, some metastatic tumors synthesize thyroid hormone in normal or even excessive amounts. RAI therapy could improve survival in these patients [46].

Occasionally the primary lesion of a follicular tumor appears to be entirely benign, but distant metastases are found. Invasion of blood vessels or the capsule, apart from the metastasis, is the only reliable criterion of malignancy. This variant has been called the benign metastasizing struma or malignant adenoma. It has a more prolonged course than do other varieties of follicular tumor, and is the type that has offered the best opportunity for the therapeutic use of ^{131}I.

The mortality attributable to follicular cancer in the 10 - 15 years after diagnosis is 30 - 50% [42, 45, 46]. Of the patients dying from the lesion, three-fourths cause death from the effect of distant metastases and the remainder from locally invasive disease.

1.7 Hurthle cell tumors

Hurthle cell tumors are histologically distinct from other follicular tumors, but they pursue a similar course. They tend to invade and metastasize locally and have a strong propensity to recur after surgery. The course tends to be prolonged. These carcinomas often do not accumulate ^{131}I. However, in a large survey, Caplan et al. [47] found that 4.4% of Hurthle cell neoplasms were hot on scan and 8.9% were warm. Serum TG levels may be normal or elevated. Cheung et al. recently studied the presence of *ret/PTC* gene rearrangements in Hurthle cell tumors and found that many expressed *ret/PTC*, and also had

other evidence of a papillary cancer origin, including focal nuclear hypochromasia, grooves, and nuclear inclusions. Tumors with the ret/PTC gene rearrangement tended to have lymph node metastases, rather than hematogenous spread. Thus Hurthle cell tumors can be classified into Hurthle cell adenomas, Hurthle cell carcinomas, and Hurthle cell papillary thyroid carcinoma [48].

1.8 Papillary thyroid carcinoma

Papillary carcinoma has a peak incidence in the third and fourth decades [41]. It occurs three times more frequently in women than in men, and accounts for 60-70% of all thyroid cancers in adults and about 70% of those found in children. The disease tends to remain localized in the thyroid gland and in time metastasizes locally to the cervical or upper mediastinal nodes. The lesions are multicentric in 20% or more of patients, especially in children. Using rigid pathologic criteria, two-thirds of predominantly papillary thyroid cancers are found to have follicular elements. The natural history of these tumors is similar to that of pure papillary lesions [49].

Papillary thyroid tumor tends to be indolent and may exist for decades without killing the patients. In a Mayo Clinic series of papillary tumors that were detected because of lymph node metastasis or found incidentally during surgery of the thyroid gland, all the patients were unaffected by the tumors over several decades [41].

The term occult has been used in a variety of ways, including reference to tumors with malignant nodes but no obvious primary, or in reference to any tumor under 1.5 cm in diameter. Mayo Clinic reports of papillary tumors under 1.5 cm in diameter, treated with conservative subtotal thyroidectomy and node dissection, have stressed their nonlethal nature, but a 1980 follow-up report on 820 patients treated by this group notes that 6 patients

eventually died after spread of tumor from such "occult" primaries [50]. Patients with appropriately treated Clinical Class I or II lesions have 96-100% survival even after 15-30 years. Survival lowers to 87% for Class III and 35% for Class IV lesions at 15 years.

While the disease may be aggressive in children, it is distinctly less aggressive in young adults, as compared to patients over age 40 [51]. Young patients tend to have small primary lesions and extensive adenopathy, but even with local invasion, but their survival is good. When papillary thyroid cancer occurs in persons over the age of 45, it may show several areas of undifferentiation, and pursue a highly malignant clinical course. The lesions tend to be larger and more infiltrative, and to have fewer local metastases [45]. It is possible that persons dying in older age actually have had their disease since youth, and that it has evolved into a more malignant phase [42, 52].

Papillary carcinoma tends to metastasize locally to lymph nodes, and occasionally produces cystic structures near the thyroid that are difficult to diagnose because of the paucity of malignant tissue. The presence of nodal metastasis correlates with recurrence [42,52,53] but has little effect on mortality in patients under age 45. In some studies, cervical adenopathy even seems to confer a protective effect on young people [42]. In patients over 45, the presence of nodes is associated with greater recurrence rates and more deaths [54, 55]. The tumors often metastasize elsewhere, especially to lung or bones.

Papillary tumors may metastasize to the lungs and produce a few nodules, or the lung fields may have a snowflake appearance throughout. These tumors are amazingly well tolerated and may allow relatively normal physical activity for 10-30 years. At times the pulmonary metastases are active in forming thyroid hormone, and may even function as the sole source of hormone supply after thyroidectomy. The metastases may progress gradually to obstructive and restrictive pulmonary disease. They also may develop arteriovenous shunts,

with hypoxia or cyanosis. Such shunts become more prominent during pregnancy, perhaps as an effect of the increased supply of estrogens.

The usual net extra mortality in papillary cancer is not great when compared to that of a control population - perhaps 10-20% over 20-30 years [42,53,55]. Mortality is rare in patients diagnosed before age 40, and is much greater in the patients found to be in clinical stages III and IV (Tables 1 and 2) at initial diagnosis (Fig. 2). However, Frazell and Duffy [56] have noted that papillary carcinoma is not always benign; they reported 35 patients with "invasive papillary carcinoma," which had a very malignant course.

1.9 The prognosis of thyroid cancer

Most thyroid cancers are very curable. In fact, the most common types of thyroid cancer (papillary and follicular) are the most curable [57]. In younger patients, both papillary and follicular cancers can be expected to have better than 95% cure rate if treated appropriately[58]. Both papillary and follicular cancers are typically treated with complete removal of the lobe of the thyroid which harbors the cancer, plus, removal of most or all of the other side.

Medullary cancer of the thyroid is significantly less common, but has a worse prognosis. Medullary cancers tend to spread to large numbers of lymph nodes very early on, and therefore requires a much more aggressive operation than does the more localized cancers such as papillary and follicular. This cancer requires complete thyroid removal plus a dissection to remove the lymph nodes of the front and sides of the neck.

The least common type of thyroid cancer is anaplastic cancer which has a very poor prognosis. It tends to be found after it has spread and is not cured in most cases. Often an operation cannot remove all the tumor.

1.10 Chemotherapy

Thyroid cancer is unique among cancers, in fact, thyroid cells are unique among all cells of the human body. They are the only cells which have the ability to absorb Iodine. Iodine is required for thyroid cells to produce thyroid hormone, so they absorb it out of the bloodstream and concentrate it inside the cell. Most thyroid cancer cells retain this ability to absorb and concentrate iodine[59]. This provides a perfect "chemotherapy" strategy. Radioactive Iodine is given to the patient and the remaining thyroid cells (and any thyroid cancer cells retaining this ability) will absorb and concentrate it. Since all other cells of our bodies cannot absorb the toxic iodine, they are unharmed. The thyroid cancer cells, however, will concentrate the poison within themselves and the radioactivity destroys the cell from within[60].

Not all patients with thyroid cancer need radioactive iodine treatments after their surgery. Others, however, should have it if a cure is to be expected. Patients with medullary cancer usually do not need iodine therapy, because medullary cancers almost never absorb the radioactive iodine. Some papillary cancers treated with a total thyroidectomy may not need iodine therapy as well. These cancers are often cured with complete surgical therapy alone[61].

II. Specific aims:

Thyroid cancer is the most common endocrine cancer and is one of the few cancers that has increased in incidence rates over the past several years. Its incidence has increased by about 3% per 100,000 people per year. The American Cancer Society estimates that there will be about 23,600 new cases of thyroid cancer in the U.S. in 2004. Of these new cases, about 17,640 will occur in women and about 5,960 will occur in men. About 1,460 people (840 women and 620 men) will die of thyroid cancer in 2004. Many patients, especially in the early stages of thyroid cancer, do not experience symptoms. However, as the cancer develops, symptoms can include a lump or nodule in the front of the neck, hoarseness or difficulty speaking, swollen lymph nodes, difficulty swallowing or breathing, and pain in the throat or neck.

There are several types of thyroid cancer: papillary, follicular, medullary, anaplastic, and variants. Papillary and follicular thyroid carcinomas are referred to as well-differentiated thyroid cancer and account for 80–90% of all thyroid cancers. Their treatment and management are similar. If detected early, most papillary and follicular thyroid cancer can be treated successfully. However, follicular thyroid cancer is a slowly growing tumor and frequently is recognized as a nodule in thyroid gland before metastases appear. Therefore, the development of more genetic or protein marker for early detection is highly desirable.

Some of gene variants have been recognized in thyroid cancer, including mutations of *h-ras* in throid adenoma and carcinoma, *retPTC* of chromosomal rearrangement in papillary cancers, overexpression of *galectin-3*, *hTERT*, *CD 97*,*VEGF* in thyroid cancer, but a question is whether these genetic changes represent the result of carcinogenesis. Thus, we adduce here a four-part experimental plan and prove that the aberrant expression of PDGFR-α is observed in follicular and papillary thyroid carcinoma cell, involves in providing a proliferation

potential in thyroid cells, statistically correlates with malignant tumor stage and finally repressed by PDGFR-α siRNA causing follicular thyroid cell to reduce proliferation (Fig. 3). In part I, we used the cDNA expression array technology to profile differentially expressed genes from human follicular thyroid carcinoma and reveal new genetic markers as well as target genes for therapeutic intervention. In Part II, to discover the over-expressed mRNA of both PDGF-A and PDGF α-receptor in thyroid carcinoma cells and imply that autocrine activation of PDGF-α receptor plays a role in the carcinogenesis of thyroid cells. In Part III, to evaluated the immunohistochemical expressions of PDGFR-α in a consecutive series of 47 resected follicular thyroid neoplasms and shows that up-regulated expression of PDGFR-α appeared in follicular thyroid carcinomas. In Part IV, to prove the phenomenon further by genetic method, RNA interference. We designed short interfering RNAs (siRNA) specific for *PDGFR-α* to repress cell proliferation in follicular thyroid carcinoma cell line(CGTH W-1), compared the effects of repressing cell proliferation of CGTH W-1 cells by PDGFR-α siRNA and a tyrosine kinase inhibitor, tyrphostin AG1295 and find that PDGFR-α siRNA required 24 hours more than tyrphostin AG1295 to show significant inhibition of cell proliferation, but the effects last up to 240 hours. These experiments provided us with a better understanding of the role of PDGFR-α on thyroid carcinogenesis.

Part I

Identifying Differentially Expressed Genes Associated with Malignance of Thyroid Cancer by Complementary DNA Expression Array

I. Abstract

Patients with follicular thyroid carcinoma had a high incidence of metastasis at the time thyroid cancer was diagnosed. Thus far, there is limited clinical factors can be used to early diagnose the presence of follicular thyroid cancer. In part 1, we used the cDNA expression array technology to profile differentially expressed genes from human follicular thyroid carcinoma and reveal new tumor markers. Tissue samples or thyroid carcinoma cell lines were obtained during surgical resection of the patients with thyroid tumor. Hybridization of identical Atlas^{TM} human cDNA expression arrays was performed with ^{32}P-labeled cDNA probes derived from RNA of thyroid cancers. Parallel analysis of the hybridized signals enables us to identify the alteration of gene expression in the malignant process. We identified 18 genes significantly over-expressed and 40 genes significantly under-expressed in the metastatic thyroid cancer. In addition, analyzing the gene expression pattern of follicular and papillary thyroid carcinoma cell lines, it was found that compared with benign tissues of thyroid nodular hyperplasia, 41 genes in CGTH W-1 exhibited more than 2-fold upregulation, while 38 genes exhibited more than 2-fold downregulation. Compared with nodular hyperplasia tissues, 35 genes in CGTH W-3 exhibited more than 2-fold upregulation, while 22 genes exhibited more than 2-fold downregulation. Genes that showed altered expression were associated with the processes of cell cycle regulation, apoptosis, DNA damage response, angiogenesis, cell adhesion and mobility, invasion, and immune response. In conclusion, we identified expression profiles of genes that are associated with malignant process of follicular thyroid cancer. Further investigation (in Part 2) is needed to understand the precise

relationship between the altered expression of these genes and the malignant process of follicular thyroid cancer.

II. Introduction

Patients with follicular thyroid carcinoma have a higher incidence of metastasis when thyroid cancer is diagnosed than patients with papillary thyroid carcinoma [1-4]. In addition, the metastasis of follicular thyroid carcinoma occurred earlier and is more likely to be associated with mortality than that of papillary thyroid carcinoma at diagnosis for a large group of patients [4]. The tumor cells must successfully complete a series of steps that include separation from the primary site, invasion into the lymphatics and/or blood stream, survival from host immunological responses, invasion into distant sites, and colonization of the new tissue or organ in order to form metastatic colonies, and several reviews have discussed the many variables associated with the cascading processes [5-8]. Understanding the process of follicular thyroid cancer malignance may lead to early and effective treatment and better prognosis.

Comparing gene expression of cells in pathological changes that arise in tumor malignance provides the underlying information related to the malignant processes [9,10]. The malignant process may include altered expression of specific genes such as those encoding proto-oncogenes, tumor suppressors, cell cycle regulatory proteins, intracellular signal transducers, apoptosis-associated proteins, DNA synthesis/repair/recombination proteins, transcription factors, cellular adhesion proteins, molecular chaperon proteins, invasion associated proteins, cytokines. Thus far, the general profiles for the altered expression of such genes in thyroid cancer cells are not available. However, the following methods can be used to identify genes that are expressed at different stages in cancer cells: subtractive hybridization, large-scale sequencing, expressed-sequence-tag analysis, serial analysis of gene expression and differential display [11-14]. These techniques produce a considerable number of cDNA representing differentially expressed genes that must be further studied. Several high throughput and hybridization-based methods have been used to quantify the expression levels of genes. Among those techniques, the cDNA expression array derived

from the “reverse-northern” dot blots technique, provides a rapid and highly effective means for high throughput screening the differential expression of many genes in pathogenic cells [15, 16].

In Part 1, the cDNA microarray technology and the appropriate thyroid samples to analyze genes that are differentially expressed in different thyroid tissues were utilized to observe complex alteration of gene expression involved in tumor malignance.

III. Materials and Methods

3.1 Tissue samples and cell cultures. Benign and malignant tissue samples were obtained during surgical resection of thyroid hyperplastic nodules and follicular thyroid carcinoma from the Department of Pathology, Chang Gung Memorial Hospital, Taiwan. The tissue specimens were frozen in liquid nitrogen and then stored at -70℃ until RNA extraction. In order to provide materials for the thyroid cancer study, Lin *et al*.[17] have built various thyroid cell lines, including CGTH W-1 (derived from metastatic follicular thyroid carcinoma) and CGTH W-3(derived from papillary thyroid carcinoma), which were obtained from the Division of Endocrinology and Metabolism, Chang Gung Memorial Hospital, Taiwan. Monolayer cultures of CGTH W-1 and CGTH W-3 were grown in RPMI medium 1640 (Gibco BRL, Life Technology) supplemented with 10% fetal calf serum, 2 g/L sodium bicarbonate (Sigma), 1% (v/v) nonessential amino acid (Gibco BRL, Life Technology), 1 mM sodium pyruvate (Gibco BRL, Life Technology), 100 U/mL penicillin G sodium, and 100 μg/mL streptomycin(Gibco BRL, Life Technology).

3.2 Total RNA isolation. Total RNA was obtained by extracting tissues and cell lines in Trizol reagent (INVITROGEN Life Technologies, Invitogen Corporation, CA) according to the manufacturer's instructions. Thyroid tissues (~100 mg each) and thyroid carcinoma cell lines (~5×10^6 cells) were homogenized in Trizol solution (1 mL). Homogenates were incubated for 5 minutes at 25℃, and then 0.2 volume of chloroform was added to the homogenates. The inorganic phase was separated by centrifugation at 12,000 g for 20 minutes at 4℃ after vigorous agitation for 5 minutes. RNA was then precipitated in the presence of 0.5 volume of isopropanol. RNA pellets were washed with 70% ice-cold ethanol and then dissolved in RNase-free water. Total RNA concentration was assessed with UV spectrophotometer (Gene Quant II, Pharmacia Biotech, Sweden) at 260 nm absorbency. RNA

quality was confirmed and visualized as 18s and 28s bands in the agarose gel without a smearing pattern (Fig. 1 & 5).

3.3 Poly A^+ RNA enrichment. Poly A^+ RNA enrichment and cDNA probe synthesis were performed from 50μg total RNA preparations by Atlas™ Pure Total RNA Labeling system(CLONTECH, CLONTECH Laboratories, Inc., CA) according to the manufacturer's instructions. The total RNA labeling system includes streptavidin-coated magnetic beads and biotinylated oligo(dT) which allow to carry out both poly A^+ RNA enrichment and probe synthesis in a single procedure. The total RNA thoroughly mix with biotinylated oligo(dT), then incubated with streptavidin-coated magnetic beads. Using the magnetic particle separator separate beads, discard supernatant. After wash the magnetic beads with poly A^+ RNA, resuspend beads in 6μl dH_2O.

3.4 Synthesis and Hybridization of cDNA Probe. cDNA probe preparation and membrane hybridization were performed according to the manufacturer's instructions for Atlas™ human cancer cDNA expression array (CLONTECH). Briefly, 1 μg polyA RNA was reverse-transcribed into cDNA by MMLV reverse transcriptase in the presence of CDS primer mix and α-^{32}P-dATP (3000 Ci/mmol, Amersham Pharmacia, Hong Kong). Labeled cDNA was purified from unincorporated nucleotides using a CHROMA SPIN-200 column (CLONTECH).

The human cDNA expression arrays were prehybridized at 68℃ for 30 minutes in ExpressHyb solution (CLONTECH) to which 0.1 mg/mL salmon sperm DNA (Gibco BRL, Invitrogen Corporation, NY) had been added. The cDNA probes were then hybridized to the arrays at 68℃ overnight. The membranes were washed 4 times with 2x SSC solution containing 1% sodium dodecyl sulfate (SDS) and twice with 0.1x SSC solution containing

0.5% SDS for 30 min at 68℃ in all cases and then exposed to a phosphor screen. The images and quantitative data of gene expression levels were analyzed with a Phosphoimager (Molecular Dynamics, Sunnyvale, Calif).

3.5 Reverse Transcription-PCR Analysis. Complementary DNA was synthesized from 2 μg of total RNA in a 25 μl reaction mixture containing 1x reverse transcriptase reaction buffer (Promega, Promega Corporation, WI), 200 μM dNTPs, 10 ng oligo $(dT)_{15}$ primer, 8 mM dithiothreithol, 40 U Rnasin (Promega) and 100 U MMLV reverse transcriptase (Promega). The mixture was incubated at 37℃ for 50 min, heated to 80℃ for 10 min, and then chilled on ice. Amplification of each specific genes was performed using a 2 μl aliquot of cDNA in a 50 μl amplification mixture containing 200 mM dNTPs, 0.2 μM forward and reverse primers, 2.5 units *Taq* DNA polymerase and 1x *Taq* reaction buffer. In the first experiment, PCR amplification for *GAPDH* was performed for 22 cycles (94°C for 30 sec, 50°C for 30 sec, 72°C for 30 sec) after a first denaturing step (94°C for 2 min). For the other genes, PCR amplification was performed for 20 to 30 cycles (94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec). The specific primers used for PCR were as follows: 5'-GTCAACGGATTTGGTCGTAT-3' and 5'-AGTCTTCTGGGTGGCAGTGA-3' for *human glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH)*; 5'-TGATGGGTTACTGTGAGCAGG-3' and 5'-GAAATC CGCTGTCTTCACACAAC-3' for *CDC-RPK*; 5'-CAAGCCCAT TCCATCCCAAC-3' and 5'-ATCACCTCCATTCACCCACC-3' for *c-fos*; 5'-CCAACTACAACTTCTTCCCTC-3' and 5'-AAGGTCCATAGCTCATCGTC-3' for *Gelatinase A*; 5'-AAAAGCAGTGTCGCCCTTCC-3' and 5'-GCCGCCTAAGTCACAAAGTC-3' for *growth hormone-dependent insulin-like growth factor-binding protein* (*IGFBP BP-53*). After amplification, the PCR products were separated in a 1.5% agarose gel and stained with ethidium bromide.

In the second experiment, the specific primers used for PCR were as follows:

5’-GTCAACGGATTTGGTCGTAT-3’ and 5’-AGTCTTCTGGGTGGCAGTGA-3’ for *human glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH)*; 5’-TTCCCCGCAATTATG TCACCCC-3’ and 5’-TTTAAATCCAACGCCCCCTCCC-3’ for *growth factor receptor-bound protein 2 isoform* (*GRB2 isoform*) ; 5’-GTGCTCCAGTAGTTTCTCAG CC-3’ and 5’-TTTCCCCTCGTTGCTCTTGTTC-3’ for *c-myc binding protein MM-1* (*c-myc MM-1*); 5’-TTGCATCATTGGCCGCACAC-3’ and 5’-TGGGCGATCCCAATTACACC AC-3’ for *cytosolic superoxide dismutase 1*(*SOD1*); 5’-AAAGTTCATGGTTCCCTGGC CC-3’ and 5’-TTGTACTGCATCCGCCGCTTAG-3’ for *fau*. After amplification, the PCR products were separated in a 1.5% agarose gel and stained with ethidium bromide.

IV. Results

4.1 RNA expression pattern in follicular thyroid carcinoma. The Atlas™ human cDNA expression array is a positively charged nylon membrane on which the DNA fragments representing 588 genes, nine housekeeping genes, and negative control sequences were spotted in duplicate dots. In the first experiment, cDNA probes derived from mRNA of primary and metastatic follicular thyroid carcinoma were hybridized to identical membrane. Several genes changed their expression levels in metastasis of follicular thyroid cancer were identified by comparing the hybridization pattern appearing on the two membranes (Fig. 2 & 3). Table 1 summarizes that 18 genes were up-regulated and 40 genes were down-regulated in metastasis process. Only those genes with larger than two-fold alterations were listed. The proteins encoded by the altered genes are associated with cell cycle regulators, growth regulators, intermediate filament markers, apoptosis, oncogenes, tumor suppressors, DNA damage responses, cell adhension and mobility, angiogenesis, invasion regulators, cell-cell interactions, immune responses. Nine housekeeping genes were used as internal controls to correct the mRNA abundance. Among these housekeeping genes, *gapdh*, *tubulin α*, β-*actin*, gene of 23-kDa highly basic protein, and gene of ribosomal protein S9, which showed similar relative intensities of signals in both hybridized membranes, were used to normalize the target genes. The signals of other housekeeping genes (*hprt*, *phospholipase A2*, and *MHC*) were either not detected or too weak to act as a useful reference. No detectable signal appeared at the sites of M13, λ-DNA, and pUC18 DNA, which served as negative control for DNA contamination of sample. In the second experiment, analyzing the gene expression pattern of follicular (CGTH W-1) and papillary (CGTH W-3) thyroid carcinoma cell lines with cDNA microarray, it was found that compared with benign tissues of thyroid nodular hyperplasia (Fig. 6 & 7), 41 genes in CGTH W-1 exhibited more than 2-fold upregulation, while 38 genes

exhibited more than 2-fold downregulation (Table 2). Compared with nodular hyperplasia tissues, 35 genes in CGTH W-3 exhibited more than 2-fold upregulation, while 22 genes exhibited more than 2-fold downregulation. The 13.4% (79 of 588) in CGTH W-1 cell line and 9.7% (57 of 588) in CGTH W-3 cell line of the gene elements were differentially regulated at an expression threshold of 2-fold difference.

4.2 RT-PCR Analysis. To confirm the differential expression of genes identified on the cDNA expression arrays, total RNAs derived from thyroid carcinoma and metastatic tissue were subjected to RT-PCR analysis for five interesting genes (*GAPDH, CDC-RPK, c-fos, Gelatinase A, IGFBP*) in the first experiment. As shown in Figure 4, these genes studied showed the same expression pattern by gene-specific RT-PCR as observed using the cDNA expression arrays. No signal was detected by RT-PCR analysis when the cDNA synthesis step was carried out without adding reverse transcriptase, indicating that the genomic DNA contamination is negligible in our analysis condition.

In the second experiment, total RNAs derived from a tissue of nodular hyperplasia, follicular thyroid carcinoma cell line and papillary thyroid carcinoma cell line were subjected to RT-PCR analysis for five interesting genes (*GAPDH, GRB2 isoform, c-myc MM-1, SOD1, fau*). As shown in Figure 8, the gene expressions of *c-myc MM-1, SOD1* showed the same expression pattern by gene-specific RT-PCR as observed using the cDNA expression arrays.

V. Discussion

Most of previous studies were undertaken to clarify biological and morphologic characteristics in human thyroid tumorigenesis. Expression genetics is a conceptually different approach to cancer diagnosis and prognosis. To understand the functional significance of specific gene products involved in tumor progression, it is practicable to define differential gene expression profiles by comparing the expression patterns of different tumor stages. RT-PCR and Northern blot analysis have been widely used for expression analysis, however, these studies are time-consuming and are only applicable to a restricted number of genes. Thus, a systematic approach for simultaneously analyzing large numbers of genes is required. Recently, the cDNA microarray technology has been developed for parallel analysis of the differential expression of specific genes in an entire cDNA population. The human cDNA expression array system provides an effective method for profiling the expression of 588 human genes in single experiment. The analytic technique is based on reverse northern blot hybridization. Each cDNA clone was immobilized in duplicate onto the nylon membrane, and each cDNA fragment length was ranged from 200 to 500 bp. The cDNA was designed without a poly-A tail, repetitive elements, and highly homologous sequences to avoid cross-hybridization and nonspecific binding. The expression pattern can be analyzed by an autoradiography after hybridization and a high-stringency wash. Seven genes (*IGFBP BP-53, Gelatinase A, c-fos, CDC-RPK, GAPDH, c-myc MM-1, SOD1*) analyzed agree in their expression pattern as observed in the Atlas human cDNA expression arrays according to the gene-specific RT-PCR technique.

Some of the genes differentially expressed in metastatic tissue, as compared with the thyroid follicular carcinoma, were some previously implicated in cancer development [18]. For example, cell cycle regulators such as cyclin-dependent kinase (CDK) and the

cyclin-dependent kinase inhibitor (CKI) are regarded as crucial contributors to cancer. In our analysis, genes of CDC2-related protein kinase (PISSLRE), CDK inhibitor p19 (INK4d), and p38 mitogen activated protein kinase displayed significantly increased expressions in metastastic tissue [18-20], indicating that activation of these gene expressions may be involved in the development of thyroid cancer metastasis. In addition, the proto-oncogene *c-fos*, may induce cell proliferation, differentiation and development [21], elevated 11-fold expression as observed in metastatic tissue (compared to thyroid follicular carcinoma). It is well known that activation of mitogen-activated protein kinases (MAPK) mediated by growth hormone-dependent insulin-like growth factor-binding protein (IGFBP) plays an important role in stimulating expression of *c-fos* gene [22-24]. Overexpressions of *IGFBP*, *p38* MAPK and *c-fos* were all observed in the metastatic thyroid tissue examined herein.

Abnormal cell surface adhesion molecules functions may lead to cancer since these molecules are involved in the regulation of cell growth and movement. Fibronectins (FN) are cell surface proteins that play a key role in the adhesive and migratory behavior of cells such as embryonic development, hemostasis, wound healing, oncogenic transformation and metastasis [25]. FN-producing cells were significantly reduced in lung metastatic tissue in the highly metastatic mouse mammary adenocarcinoma assay [26]. Our detection of decreased levels of FN in metastatic tissue of thyroid follicular cancer also proved that FN expression was reduced in invasive tumors.

The second investigation could let us find which candidate genes were suitable to be tumor markers in follicular thyroid carcinoma. Among these candidate genes(Table 2), we find the *copper-zinc superoxide dismutase* (*SOD1*) reducing ~2.7 folds expression in follicular thyroid carcinoma cell line. In previous research, lowered amount of the copper-zinc-containing superoxide dismutase have been found in many tumors, but not all. Superoxide dismutases (SOD) are essential enzymes that eliminate superoxide radical(O^{2-}) and thus protect cells from damage induced by free radicals[27,28]. The active O^{2-} production

and low SOD activity in cancer cells[29,30] may render the malignant cells highly dependent on SOD for survival and sensitive to inhibition of SOD. Inhibition of SOD causes accumulation of cellular O^{2-} and leads to free-radical-mediated damage to mitochondrial membrances, the release of cytochrome c from mitochondria and apoptosis of the cancer cells. [31].

We demonstrate that the cDNA array technique is an effective tool for monitoring the overall profile of gene expression in thyroid tumorigenesis. The profile of gene expression in thyroid neoplasm will help to reveal genes that commonly expressed in this type of tumor. Unless more clinical cases are studied, the result was not representative to all follicular thyroid carcinoma and their malignance. Therefore, we performed the related experimental plan in Part 3 of the thesis. In addition, determining how relevant the expression difference of a particular gene is to the underlying oncogenic or metastatic process remains problematic. Therefore, we examined the malignant process in Part 2 of the thesis. However, information from these studies may help develop diagnosis methods and therapeutic drugs for the thyroid cancers.

Part 2

An Aberrant Autocrine Activation of the Platelet-derived Growth Factor α-receptor in Follicular and Papillary Thyroid Carcinoma Cell Lines

I. Abstract

Platelet-derived growth factor receptor (PDGFR) can bind to its ligand and consequently possesses a kinase activity, and which is associated with the carcinogenesis of different cell types, including astrocytomas, oligodendrogliomas, and glioblastoma. In a cDNA microarray analysis, we observe the over-expressed mRNA of both PDGF-A and PDGF α-receptor in thyroid carcinoma cells (in Part 1). Then, the elevated protein expressions of PDGF-A and PDGF α-receptor in thyroid carcinoma cells were also confirmed by a Western blot analysis. The phosphorylation of PDGF α-receptor evaluated by an antibody against Tyr 720-phosphate was found in thyroid carcinoma cells. The tyrosine kinase activity of PDGF α-receptor was inhibited by tyrphostin AG1295 and showed a dose-dependent inhibition for the proliferation of thyroid carcinoma cells. These findings imply that autocrine activation of PDGF-α receptor plays a crucial role in the carcinogenesis of thyroid cells.

II. Introduction

Platelet-derived growth factor (PDGF) consists of A- and B-polypeptide subunits and arranges as PDGF-AA, PDGF-AB, and PDGF-BB. These isoforms have different specificities and affinities to the PDGF α- and β-receptors. The PDGFR-α has a high affinity for all isoforms, while the PDGFR-β has a high affinity only for PDGF-BB. Both α-receptor and β-receptor belong to the same family of receptor proteins as the *c-fms* [1] and *c-kit* [2,3] proto-oncogene families, and possess activity of tyrosine kinase after stimulated by ligands binding. Expressions of PDGF and PDGFRs have crucial functions during the embryogenesis, in particular for the development of connective tissue of the kidneys, blood vessels, lungs, and the central nervous system [4–6]. In addition, PDGF and cognate receptors are also important during the formation of connective tissue of wound healing in the adult [7]. Besides the normal functions of PDGF and cognate receptor, other reports indicated that the excess activity of PDGFR caused by abnormal binding of cognate ligands and consequently stimulating the signaling pathway was associated with different disorders, such as glioblastoma and sarcoma [8]. Ectopic autocrine stimulation caused by abnormal expression of PDGF and cognate receptor was also associated with atherosclerosis and various fibrotic conditions, including lung fibrosis, kidney fibrosis, liver cirrhosis, and myelofibrosis [9–11].

Thyroid carcinomas are classified pathologically as papillary, follicular, or anaplastic carcinoma of thyroid follicular epithelial cell origin and as medullary carcinoma of parafollicular cell origin. PDGFRs mainly exist in mesenchyme-derived and glia-derived cells, but not in normal epithelial cells [12, 13]. With regard to the PDGF and cognate receptor expression in thyroid cells, previous findings show that normal thyroid cells possess receptors for epidermal growth factor (EGF), though all lack PDGF binding sites [12]. However, the presence of PDGFR-β was found in human anaplastic thyroid carcinoma cell line C643 [14]. In another study, the expression of PDGFR-α and -β were found in human

anaplastic thyroid carcinoma cell line HTh74 [15]. Those findings indicate that the expression of PDGFRs provides the cells a new growth stimulation route and play a crucial role in the carcinogenesis of thyroid cells. Another possibility, the expression of PDGFRs may only be the remnants of immature progenitor cells.

In Part 1, we found that mRNA of PDGF-A and PDGFR-α were highly expressed in thyroid carcinomas but not in nodular hyperplasia cells by a cDNA microarray technique. These results cause the motive to investigate whether autocrine activation caused by abnormal expression of PDGF and cognate receptor exists in thyroid cells, and whether play a crucial role in carcinogenesis. In Part 2, we examined the expression of PDGF-A and PDGFR-α, phosphorylation activation of PDGFR-α, and whether PDGF-related autocrine activation is a critical event in cell proliferation of thyroid carcinoma. Information from the present study led to the better understanding of the involvement of PDGF-related anutocrine activation in thyroid carcinogenesis.

III. Materials and methods

3.1. Tissue samples and cell cultures. Benign and malignant tissue samples were obtained during surgical resection of thyroid hyperplastic nodules and follicular thyroid carcinoma from the Department of Pathology, Chang Gung Memorial Hospital, Taiwan. The tissue specimens were frozen in liquid nitrogen and then stored at -70℃ until RNA or protein extraction.

In order to provide materials for the thyroid cancer study, Lin *et al.*[16] have established various thyroid cell lines, including CGTH W-1 (derived from metastatic follicular thyroid carcinoma) and CGTH W-3 (derived from papillary thyroid carcinoma), which were obtained from the Division of Endocrinology and Metabolism, Chang Gung Memorial Hospital, Taiwan. Monolayer cultures of CGTH W-1 and CGTH W-3 were grown in RPMI medium 1640 (GIBCO, Invitrogen Corporation, NY) supplemented with 10% fetal calf serum, 2 g/L sodium bicarbonate (SIGMA, Sigma-Aldrich, MO), 1% (v/v) nonessential amino acid (GIBCO), 1 mM sodium pyruvate (GIBCO), 100 U/mL penicillin G sodium, and 100 μg/mL streptomycin(GIBCO).

3.2. Total RNA isolation. Total RNA was obtained by extracting tissues and cell lines in Trizol reagent (INVITROGEN Life Technologies, Invitogen Corporation, CA) according to the manufacturer's instructions. Thyroid tissues (~100 mg each) and thyroid carcinoma cell lines (~5×10^6 cells) were homogenized in Trizol solution (1 mL). Homogenates were incubated for 5 minutes at 25℃, and then 0.2 volume of chloroform was added to the homogenates. The inorganic phase was separated by centrifugation at 12,000 g for 20 minutes at 4℃ after vigorous agitation for 5 minutes. RNA was then precipitated in the presence of 0.5 volume of isopropanol. RNA pellets were washed with 70% ice-cold ethanol and then dissolved in RNase-free water. Total RNA concentration was assessed with UV

spectrophotometer (Gene Quant II, Pharmacia Biotech, Sweden) at 260 nm. RNA quality was confirmed and visualized as 18S and 28S rRNA bands in the agarose gel without a smearing pattern.

3.3. Synthesis and hybridization of cDNA probe. cDNA probe preparation and membrane hybridization were performed according to the manufacturer's instructions for Atlas™ human cancer cDNA expression array (CLONTECH, CLONTECH Laboratories, Inc., CA). Briefly, 1 μg polyA RNA was reverse-transcribed into cDNA by MMLV reverse transcriptase in the presence of CDS primer mix and α-^{32}P-dATP (3000 Ci/mmol, Amersham Biosciences, Amersham Biosciences Ltd, Hong Kong). Labeled cDNA was purified from unincorporated nucleotides using a CHROMA SPIN-200 column (CLONTECH).

The human cDNA expression arrays were prehybridized at 68℃ for 30 minutes in ExpressHyb solution (CLONETECH) to which 0.1 mg/mL salmon sperm DNA (Gibco BRL, Invitrogen Corporation, NY) had been added. The cDNA probes were then hybridized to the arrays at 68℃ overnight. The membranes were washed 4 times with 2x SSC solution containing 1% sodium dodecyl sulfate (SDS) and twice with 0.1x SSC solution containing 0.5% SDS for 30 min at 68℃ in all cases and then exposed to a phosphor screen. The images and quantitative data of the gene expression levels were analyzed with a Phosphoimager (ImageQuaNT, Molecular Dynamics, CA).

3.4. Quantitative PCR analysis. Total RNA from thyroid carcinoma cell lines or thyroid tissues derived from nodular hyperplasia was extracted by using Trizol reagent (INVITROGEN) according to the manufacturer's instructions. For reverse transcription, equal amount of total RNA (2 μg)were performed in a 25-μl reaction mixture containing 1x reverse transcriptase reaction buffer (Promega, Promega Corporation, WI), 200μM dNTPs, 10ng

oligo $(dT)_{15}$ primer, 8mM dithiothreithol, 40 units Rnasin (Promega), and 100 units MMLV reverse transcriptase (Promega). The mixture was incubated at 42℃ for 50 minutes , heated to 70℃ 10 minutes, and then chilled on ice. The GeneAmp 5700 sequence detection system (Applied Biosystems, Applied Biosystems, CA) was used to amplify both target genes (PDGF-A and PDGFR-α) and internal control (β-actin). The reaction master mix was prepared according to the manufacture's protocol to give final concentration of 1x SYBR Green PCR buffer, 3mM $MgCl_2$, dNTP blend(0.2mM dATP, 0.2mM dCTP, 0.2mM dGTP, 0.4mM dUTP), 0.025 units AmpliTaq Gold DNA polymerase, 0.01 units AmpErase uracil-N-glycosylase, and 300nM primers. The specific primers for PCR were as follows: 5'-CACGC CACTA AGCAT GTGCC-3' and 5'- ATGAC CGTTC CTGGT CTTGC AG-3' for PDGF-A, GenBank accession number: X06374; 5'-TGAAG AAAAC AACAG CGGCC-3' and 5'-CGTCA TTCCT AGAGG TACAA AGGCT-3' for PDGFR-α, GenBank accession number: M21574; 5'-ATGGG TCAGA AGGAT TCCTA TGTG-3' and 5'-GCCAG ATTTT CTCCA TGTCG TC-3' for β-Actin, GenBank accession number: X00351. Complementary DNA synthesized by reverse transcription was added to the master mix. Then, the PCR reagent mix were transferred to thermocycler and PCR profile were performed at 50℃ for 2 min, 95℃ for 10 min, and followed by 40 cycles of amplification at 95℃ for 15 sec, 60℃ for 1 min, using the GeneAmp 5700 sequence detection system.

Relative expression of PDGF-A or PDGFR-α transcripts was determined by the following calculation, as described in the Applied Biosystems users bulletin, 'Relative Quantitation of Gene Expression':

Relative expression = $2^{-\triangle\triangle Ct}$

Where $\triangle\triangle Ct_{\text{PDGF-A}} = (Ct_{\text{PDGF-A}} - Ct_{\beta\text{-Actin}})_{\text{thyroid carcinoma cell line}} - (Ct_{\text{PDGF-A}} - Ct_{\beta\text{-Actin}})_{\text{nodular hyperplasia}}$

or $\triangle\triangle Ct_{\text{PDGFR-}\alpha} = (Ct_{\text{PDGFR-}\alpha} - Ct_{\beta\text{-Actin}})_{\text{thyroid carcinoma cell line}} - (Ct_{\text{PDGFR-}\alpha} - Ct_{\beta\text{-Actin}})_{\text{nodular hyperplasia}}$

3.5. Western blot analysis. Whole cell protein extracts were prepared by using cold lysis buffer consisting of 1x PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mg/mL PMSF, and 0.2 units/mL aprotinin (Santa Cruz Biotechnology, Santa Cruz Biotechnology, Inc., CA). Samples were incubated on ice for 30 minutes and supernatants were recovered by centrifuging at 10,000 g at 4℃ for 10 minutes. Protein concentrations were determined by DC^{TM} protein assay method (BIO-RAD, Bio-Rad Laboratories, Inc., CA). Proteins were separated on 10% SDS-PAGE and transferred to PVDF membrane (Amersham Biosciences). Blocking reagent was 3% gelatin in TBS (pH 7.4). The washing buffer consisted of TBS (pH 7.4) with 0.1% Tween-20. Mouse monoclonal IgG_{2b} anti–PDGF-A (E-10) (Santa Cruz Biotechnology) and rabbit polyclonal IgG anti-PDGFRα (C-20) (Santa Cruz Biotechnology) were used as primary antibodies. Goat anti-mouse IgG conjugated by alkaline phosphatase (Santa Cruz Biotechnology) and goat anti-rabbit IgG conjugated by horseradish peroxidase (Santa Cruz Biotechnology) were used as respective secondary antibodies. Signals were respectively visualized by using BCIP/NBT substrate (SIGMA) and TMB membrane peroxidase substrate (KPL, Kirkegaard & Perry Laboratories, Inc., MD).

3.6. Immunoprecipitation and western blot for PDGF α-receptor. For immunoprecipitation of PDGF α-receptor, $2x10^7$ cells were washed in 1xPBS and lysed in ice-cold RIPA buffer consisting of 1x PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mg/mL PMSF, and 0.2 U/mL aprotinin (Santa Cruz Biotechnology). Samples were incubated on ice for 30 minutes and supernatants were recovered by centrifuging at 10,000 g at 4℃ for 10 minutes. After centrifugation, 1 mL cell lysates was incubated with 2 μg rabbit polyclonal IgG anti-PDGFRα (C-20) for 2 hours at 4℃. Then precipitation was performed by using 20 μL protein A/G plus-agarose (0.5 mL agarose/2.0 mL; Santa Cruz Biotechnology) at 4℃ overnight. The precipitates were washed 3 times with PBS, extracted by adding reducing

SDS sample buffer and incubated for 5 minutes at 95℃.

Samples were analyzed on 10% SDS-PAGE and transferred to PVDF membranes (Amersham Biosciences). Blocking reagent was 3% gelatin in TBS (pH 7.4). Washing buffer consisted of TBS (pH 7.4) with 0.1% Tween-20. Goat polyclonal IgG specific for Tyr-754-phosphorylated PDGFR-α [p-PDGFR-α (Tyr754); Santa Cruz Biotechnology] and goat polyclonal IgG specific for Tyr-720-phosphorylated PDGFR-α [p-PDGFR-α (Tyr720); Santa Cruz Biotechnology] were used as primary antibodies. Anti-goat IgG-HRP (Santa Cruz Biotechnology) was used as secondary antibody. Signals were visualized by using TMB membrane peroxidase substrate (KPL).

3.7. Cell proliferation assays. CGTH W-1 or CGTH W-3 cells (2.5×10^4 per 35-mm dish) were seeded in RPMI-1640 medium with 1% fetal calf serum. Tyrphostin AG1295 (AG1295; selective inhibitor for the PDGF-receptor tyrosine kinase activity) was used at final concentrations of 0, 0.25, 0.5, 1, and 2.5 μM (Calbiochem, Merck Biosciences, Germany), and Tyrphostin A1 (AG9; negative control) was used at a final concentration of 2.5μM (Calbiochem). Triplicate dishes were used for each concentration point. Tyrphostin AG1295 and Tyrphostin A1 in RPMI-1640 media containing 1% fetal calf serum were added to the cells every other day. The cell number per dish was determined at day 7 after plating, when cells were trypsinized, suspended, and counted using a Coulter counter (Coulter Electronics, Herpendon, UK).

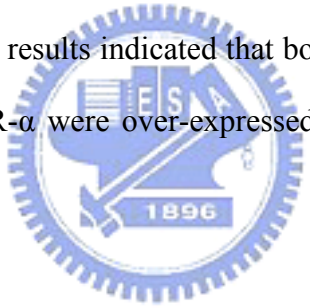
IV. Results

4.1. Expression of PDGF-A and PDGF α-receptors in thyroid carcinoma cells. By comparing the gene expression pattern of CGTH W-1 and CGTH W-3 cell lines with benign tissues of thyroid nodular hyperplasia, we identified 41 over-expressed genes and 38 suppressed genes in CGTH W-1 cell line with more than 2-fold expression difference. We also identified 35 over-expressed genes and 22 suppressed genes in CGTH W-3 (Part 1 table 2). The differences of gene expression between nodular hyperplasia and thyroid carcinoma cell lines involve large numbers of genes, and the overall profiles of gene expression were represented graphically in Figure 1. The correlation between gene expressions in CGTH W-1 cell and those in nodular hyperplasia (R^2=0.48) is very similar to that between gene expressions in CGTH W-3 cell and those in nodular hyperplasia (R^2=0.46). The greater the similarity between the gene expression patterns the more linear the dot plot graph. From figure 1, it showed that a number of candidate genes were obviously altered in thyroid carcinoma cell lines. Among these candidate genes, PDGF-A exhibited 2- and 2.7-fold over-expression in CGTH W-1 and CGTH W-3, respectively, and PDGFR-α exhibited 13- and 137.5-fold over-expressed in CGTH W-1 and CGTH W-3, respectively (Part 1 table 2). We did not observe any expression of PDGF-A and PDGFR-α in nodular hyperplasia. On the other hand, PDGF-B did not show any obvious expression among in nodular hyperplasia, CGTH W-1, and CGTH W-3. PDGF β-receptor shows no obvious expression in both nodular hyperplasia tissues and CGTH W-1 cell line, and only slight expression in CGTH W-3 cell line (Fig. 2).

To confirm the differential expression of genes identified on the cDNA expression arrays, the total RNAs derived from thyroid carcinoma cell line and nodular hyperplasia was subjected to reverse transcription and quantitative PCR for PDGF-A and PDGFR-α. Figure 3 illustrated that these gene expressions amplified by gene-specific quantitative PCR displayed

the same tendency as observed by the cDNA expression arrays. No signal was detected by quantitative PCR analysis when the cDNA synthesis step was performed without adding reverse transcriptase or the quantitative PCR step was performed without adding template (no template control; NTC), indicating that genomic DNA contamination and cross-contamination is negligible in our analysis condition.

The protein level of PDGF-A and PDGFR-α were detected by antibodies against an epitope of PDGF-A (135-211 amino acids) and PDGFR-α (carboxyl terminus), respectively. The result shows that no obvious band was detected in nodular hyperplasia, while a band of approximately 26 kDa was detected in CGTH W-1 and CGTH W-3 (Figure 4A). The polyclonal antibody against the PDGFR-α shows that marked bands around at 180, 156, 130, 90 and 52 kDa in the western blotting analysis of total protein lysate of CGTH W-1 and CGTH W-3 (Figure 4B). These results indicated that both the transcriptional and translational levels of PDGF-A and PDGFR-α were over-expressed in CGTH W-1 and CGTH W-3 cell lines.



4.2. Phosphorylated activation of the PDGFR-α in thyroid carcinomas cells. After the expression of PDGF-A and PDGFR-α in CGTH W-1 and CGTH W-3 cell lines were confirmed, we survey the PDGFR-α activity in terms of phosphorylation. Phosphorylation was detected by a western blotting with phosphor-specific antibodies. The results showed that several bands (170, 156, 120, 90 and 52KDa) were observed in CGTH W-1 and CGTH W-3 cell lines by using antibodies against p-PDGFR-α (Tyr720) (Figure 5, lanes 3 and 4). While no obvious band was observed in same cell lines by using antibodies against p-PDGFR-α (Tyr754) (figure 5, lanes 1 and 2).

4.3. The proliferation of thyroid carcinoma cells is dependent on PDGFR activation.

Tyrphostin AG1295 is an inhibitor that decreases the activity of protein tyrosine kinase. It selectively inhibits PDGFR kinase (IC_{50}=0.5μM) and PDGF-dependent DNA synthesis (IC_{50}=2.5μM), while it has no influence on EGF-receptor autophosphorylation and only a slight influence on EGF- or insulin-stimulated DNA synthesis [17,18]. When different concentrations of tyrphostin AG1295 were added to the cell lines, the result showed a dose-depend inhibition for cell proliferation. After the 7-day, the average rate of inhibiting cell proliferation obtained from triplicate experiments with adding different concentrations of tyrphostin AG1295 (0, 0.25, 0.5, 1, and 2.5 μM) were 0%, 7%, 42%, 51%, and 82% in CGTH W-1 cells(Figure 6A) and 0%, 13%, 29%, 36%, and 51% in CGTH W-3 cells(Figure 6B). In a negative control study, tyrphostin A1 did not affect cell proliferation of CGTH W-1 and CGTH W-3 cells.

V. Discussion

It has been reported that the signal transduction route induced by PDGF and cognate receptor can cause transformation and malignant tumors in experimental systems [19-22]. Then, a question aroused regarding whether PDGF and PDGFR play a role in developing spontaneous carcinogenesis in humans. A number of tumor types have been found to be related to their expression of PDGF or PDGFRs [23–26]. If the expression of PDGF and its cognate receptor are found in tumor cells, then it is possible that autocrine stimulation may exist to develop carcinoma cells. By using a cDNA microarray technique, we identified the aberrant expression of PDGF-A and PDGFR-α presented in both follicular thyroid carcinoma (CGTH W-1) and papillary thyroid carcinoma (CGTH W-3) cell lines. In previous studies, it has been found that structural aberrations of the PDGF and cognate receptor genes would lead to over-expression or expression of an abnormal protein [27-29]. Moreover, amplification of PDGFR-α gene could cause receptor over-expression in a few cases of glioblastoma [30-32]. These findings may imply that some variation of gene regulations lead to over-express the PDGF and PDGFR in CGTH W-1 and CGTH W-3 cells.

Generally speaking, normal thyroid cells do not exhibit PDGF and PDGFRs, but which are mainly exhibited in mesenchyaml and glial origin tissues. Therefore, it is unusual to observe the expression of these genes in thyroid cells. Some studies have shown that PDGFR-β can be found in human anaplastic thyroid carcinoma cell line C643 (a kind of undifferentiated thyroid cell line) [14], and that both α- and β-type PDGFRs are expressed in human anaplastic thyroid carcinoma cell line HTh74[15]. Two possibilities can be inferred from such findings. One is that the expression of PDGFR provides the cells a new route that stimulates the growth of the cells, and in this way PDGF-receptors take part in the carcinogenesis of thyroid cells. Alternatively, PDGFRs may be the remnants of immature progenitor cells.

CGTH W-1 and CGTH W-3 cell lines were obtained from well-differentiated thyroid tumor tissues. In these two cell lines, the expressions of PDGF-A and PDGFR-α proteins were observed. A segment of approximately 26 kDa was observed for PDGF-A by a western blotting analysis, while segments around at 180, 156, 130, 90, and 52 kDa were observed for PDGFR-α. Similarly sized protein segments had been observed in glioma; these segments have been inferred to be derived from full-length receptor [33]. Huang and Huang [34] confirmed that after PDGFR-β binding with PDGF-B, the receptor would quickly decompose. And it was also found that PDGF and receptor binding induces internalization of the complex into endosomes [35]. The PDGF-receptor complex then dissociates and recycles to the cell membrane, or alternatively the ligand-receptor complex is degraded after fusion of the endosomes with lysosomes. In addition to degradation in lysosomes, PDGF receptors also undergo cytoplasmic degradation in proteasomes after ubiquitination[36,37]. In the present study, we have similar observations in CGTH W-1 and CGTH W-3 cell lines regarding simultaneously expression of ligands and receptors and degradation of PDGFR-α. These observation illustrates that PDGF-A and PDGFR-α should form complex, induce internalization and undergo cytoplasmic degradation. These findings indicate that the initiation event of PDGF antocrine activation and PDGFR degradation existed in CGTH W-1 and CGTH W-3 cell lines.

When PDGF binding, it would induce the dimerization and autophosphorylation of PDGFR. The autophosphorylation has two important functions. On one hand, phosphorylation of a conserved tyrosine residue inside the kinase domains leads to an increase in the catalytic efficiencies. On the other hand, phosphorylation of tyrosine residues located outside the kinase domain creates docking sites for signal transduction molecules. We survey the PDGFR-α activation in terms of phosphorylation and found that phosphorylation of PDGFR-α in CGTH W-1 and CGTH W-3 cell lines were detected on Tyr-720, but not on Tyr-754. The result was similar to the finding that Tyr-754 in the α-receptor was

phosphorylated to a higher degree in the heterodimer compared with the homodimer [38]. Furthermore, it have been found that Tyr-720 in the PDGFR-α is required for binding of Grb2 and SHP-2 but not for activation of Ras or cell proliferation [39]. SHP-2 is a tyrosine phosphatase with two SH2 domains [40] and plays a diverse modulator. In previous studies, SHP-2 is a potential negative modulator of PDGF-related signal transduction by dephosphorylating autophosphorylated PDGFRs and substrates for the PDGF receptors [41]. However, SHP-2 may also be involved in positive signaling to act an adaptor that binds Grb2/Sos and thus to contribute to Ras activation [42], and consequently to dephosphorylate the COOH-terminal tyrosine residue of Src and thus to contribute to Src activation [43,44]. Our data reveal that homodimeric PDGFR-α would cause autophosphorylation of tyrosine 720 in CGTH W-1 and CGTH W-3 cells. However, the possible role of tyrosine kinase phosphorylation at tyrosine 720 concerning PDGFR-α signal transduction must be further studies and another phosphorylation sites should be further discovered.

The growth of certain human glioma cells were blocked by PDGF antagonists [45, 46]. In cell proliferation assay, we demonstrated that the activity of PDGFR-α tyrosine kinase was necessary for CGTH W-1 and CGTH W-3 cell proliferations. By adding tyrphostin AG1295, which specifically inhibits the activity of PDGFR kinase, we observed a dose-related inhibition of cell proliferation. These findings indicate that CGTH W-1 and CGTH W-3 cell lines have a PDGFR- related stimulation pathway, and which stimulates cell proliferation and consequently develops carcinogenesis. Using tyrphostin AG1295 as a proof for autocrine PDGF/PDGFR activity have preliminarily been observed. Furthermore, RNA interference, a process of homology-dependent degradation of cognate mRNA by short-interfering RNA (siRNA), have been showed in Part 4 of the thesis. The experiments by using RNAi to perturb PDGFR-α expression would reconfirm that thyroid carcinoma cell lines were growth dependence on the change of PDGFR-α expression, estimate the efficiency of PDGFR-α siRNA in suppress the aberrant proliferation and may provide a new tool for repressing cell

proliferation of thyroid tumor.

In conclusion, both the PDGF-A and PDGFR-α were over-expressed in the follicular thyroid carcinoma (CGTH W-1) and papillary thyroid carcinoma (CGTH W-3) cell lines. The over-expression of PDGF-A and PDGFR-α genes might be an indication of carcinogenesis. In thyroid cells, those aberrant expressions could develop an abnormal signal transduction route by the PDGF-related autocrine activation and consequently enhanced cell proliferation.

Part 3

Up-regulation of platelet-derived growth factor α-receptor expression in follicular thyroid carcinoma correlates with malignant tumor stage

I. Abstract

Platelet-derived growth factor α-receptor (PDGFR-α) belongs to the family of proto-oncogenes involved in sustaining cell proliferation. The study of Part 3 evaluated the immunohistochemical expressions of PDGFR-α in a consecutive series of 47 resected thyroid tumors. The immunohistochemistrical result showed that PDGFR-α expression was upregulated and positive in 20/36 (55.5%) cases of follicular thyroid carcinomas. The nonparametric Kruskal-Wallis statistical analysis showed a statistically significant correlation between positive PDGFR-α expression and tumor stage(P=0.01; evaluating staining intensity and P=0.001;evaluating the percentage of staining cells). These findings imply that up-regulation of PDGFR-α function may be an important step in thyroid progression. In addition, a genetic-specific siRNAs against PDGFR-α was designed and generated for specifically inhibits PDGFR-α expression and could be extended to study PDGFR-related signal transduction and understand the role for PDGFR-α in thyroid malignancy.

II. Introduction

Certain studies on oncogenes have provided much useful information on thyroid tumorigenesis. Simian virus 40-like sequences are found in many thyroid cancers and the Tag gene sequence is known to be oncogenic in animal models [1]. Overexpression of many other genes, including galectin-3 [2], Thymosin beta-10[3], hTERT [4], CD97[5], CD26[6], VEGF[7], has been detected in different thyroid neoplasm.

The human PDGFR-α gene is localized on chromosome 4q12, close to the genes for the SCF receptor and VEGF receptor-2 [8]. The PDGFR-α gene possesses molecular sizes of ~170kDa and exert their effects on target cells by activating intracellularly protein tyrosine kinase. When ligand-induced PDGF receptor activation causes autophosphorylation of the receptors, which leads to increased catalytic activity of the kinases and to the formation of docking sites for downstream signal transduction molecules containing SH2 domains. Then, the PDGFR-related signal pathway could lead to different cellular responses, including stimulation of cell growth [9,10], effects on chemotaxis [11-13], and Ca^{2+} mobilization [14].

It has been shown that abnormal PDGFR-related signal transduction is involved in the development of several serious disorders, including certain malignancies, atherosclerosis and various fibrotic conditions. Furthermore**,** several studies have found that aberrant expression of PDGFR-α was associated with several tumors[15-18] and up-regulated expression of the PDGFR-α correlated with an elevation of metastatic potential of cell lines derived from lung tumors and ovarian carcinomas[19-21].

Thyroid carcinomas are classified pathologically as papillary, follicular, or anaplastic carcinoma of thyroid follicular epithelial cell origin and as medullary carcinoma of parafollicular cell origin. PDGFRs mainly exist in mesenchyme-derived and glia-derived cells, but they are not normally found in epithelial cells [22, 23]. In Part 3, we examined the expression patterns of PDGFR-α in a consecutive series of follicular thyroid carcinomas and nodular hyperplasia. In order to elucidate the clinical significance of PDGFR-α expression,

the study was performed by semi-quantifying the expression of PDGFR-α in human thyroid cancer tissue and by correlating them with clinicopathological factors. Then, newly-designed siRNAs against PDGFR-α was used to suppressed PDGFR-α expression in the thyroid follicular carcinoma cell line (CGTH W-1) , that could provide useful information about understanding the role for PDGFR-α in thyroid malignancy.

III. Materials and methods

3.1 Patients and Cell cultures. A total of 47 patients with thyroid neoplasm, who had undergone thyroidectomy at the Department of Pathology, Chang Gung Memorial Hospital, Taiwan, were included in this study. They were all proved to have nodular hyperplasia or follicular thyroid carcinomas (FTC) by biopsies. TNM classification and clinical stage classifications of thyroid cancer were used in this study. The clinicopathological characteristics of this series are summarized in Table I.

CGTH W-1 (derived from metastatic follicular thyroid carcinoma) cell line was obtained from the Division of Endocrinology and Metabolism, Chang Gung Memorial Hospital, Taiwan. Monolayer cultures of CGTH W-1 was grown in RPMI medium 1640(GIBCO, Invitrogen Corporation, NY) supplemented with 10% fetal calf serum, 2 g/L sodium bicarbonate (SIGMA, Sigma-Aldrich, MO), 1% (v/v) nonessential amino acid (GIBCO), 1 mM sodium pyruvate (GIBCO), 100 U/mL penicillin G sodium, and 100 μg/mL streptomycin(GIBCO).

3.2 Immunohistochemistry. A standard immunoperoxidase technique was used to evaluate PDGFR-α protein expression in paraffin-embedded tissue samples. The paraffin-embedded sections from tissues of 47 patients, including 36 cases of follicular thyroid carcinoma and 11 cases of non-malignant thyroid nodular hyperplasia(NH), were stained for PDGFR-α antigen after antigen retrieval performed with 0.01M citrate solution, pH6. Briefly, deparaffinized sections were heated in a water bath for 10 min. After endogenous peroxidase was quenched with 1% hydrogen peroxide in the section, each section was incubated with 1.5% nonimmuned goat serum. Then, the sections were incubated in rabbit polyclonal IgG anti-PDGFRα (C-20; Santa Cruz Biotechnology, Santa Cruz Biotechnology, Inc., CA)at 2 μg/ml in PBS with 1.5% normal blocking serum at 4℃ overnight. Negative controls also

included performing the staining procedure but omitting the primary antibodies. The sections were incubated with biotin-conjugated secondary antibody and avidin-biotin enzyme reagent. For visualization of PDGFR-α protein expression, sections were incubated with diaminobenzidine tetrahydroxychloride (DAB) solution as the substrate. The sections were lightly counterstained with hematoxylin, rehydrated in a graded series of ethanol, cleared in xylene and coverslipped.

3.3 Immunohistochemistry evaluation. A semi-quantitative analysis of the sections was performed. Two independent observers evaluated staining intensity (graded from 0 to 3) and the percentage of positive cells (graded by 0%, 1~25%, 26~50%, 51~75% and 76~100% stained cells) after inspection of multiple fields at high magnification. Sections with a staining intensity of 2 or greater or more than 25% positive cells were considered positive for PDGFR-α expression, respectively.

3.4 Statistical analysis. The data concerning protein expression were presented as positive (> 2 or >25%) or negative. The nonparametric Kruskal-Wallis test was used to analyse the association between protein expression and clinicopathological parameters including tumor stage, lymph nodal stage, clinical stage, cell differentiation and metastasis. The $P < 0.05$ was considered to be statistical significant. Statistical analysis was performed using the SPSS/Windows 11.0 statistical package(SPSS, Inc., IL).

3.5 RNA interference. Specific siRNA oligos targeting PDGFR-α mRNAs were designed as indicated from Qiagen by using the online siRNA design tool (www.qiagen.com/sirna). Specific and control oligos were synthesized by Qiagen and the oligos were: PDGFR-α sence RNA 5'-GGCAC GCCGC UUCCU GAUA TT-3', PDGFR-α antisence RNA 5'-UAUC

AGGAA GCGGC GUGCC TT-3' ,control siRNA sence RNA :5'-UUCUC CGAAC GUGUC ACGU TT-3' and control siRNA antisence RNA: 5'-ACGU GACAC GUUCG GAGAA TT-3'. Cells were transfected using Lipofectamine 2000 (INVITROGEN life technologies, Invitrogen Corporation, CA) and samples were taken at the indicated time points.

3.6 Immunofluorescence cell staining. 10^5x CGTH W-1 cells were added to each well containing a glass slide of six-wells plate. The slides were incubated with normal serum of the same species as the second antibody to block nonspecific antibody staining. Rabbit polyclonal IgG anti-PDGFRα (C-20) (Santa Cruz Biotechnology) were applied as primary antibodies at 2 μg/ml in PBS with 1.5% normal blocking serum. Goat anti-rabbit IgG conjugated by fluorescein isothiocyanate (Santa Cruz Biotechnology) were used as secondary antibody diluted to 1 μg/ml in PBS with 1.5% normal blocking serum in dark chamber. Cell nuclei was counter-stained in DAPI. The slides were mounted coverslips with aqueous mounting medium and examined using a fluorescence microscope (Nikon TE300 inverted microscope, Nikon corporation, Japan).

IV. Results

4.1. Localized pattern of PDGFR-α in the follicular thyroid carcinoma. To identify whether the aberrant expression of PDGFR-α was related to the advanced malignant thyroid cells. The protein expression of PDGFR-α was investigated in 36 follicular thyroid carcinoma (7 FTC clinical stage I, 9 FTC stage II, 9 FTC stage III, 11 FTC stage IV) and 11 non-malignant thyroid nodular hyperplasia(NH) tissues by immunohistochemistry. The immunohistochemistrical results show that up-regulated expression of PDGFR-α appeared in follicular thyroid carcinoma, for instance, FTC clinical stage III specimen shows an aberrant expression of PDGFR-α appearing around spherical thyroid follicles (Fig. 1A), the section derived from follicular thyroid carcinoma with bone metastases shows irregular cell distribution and moderate mottled signal (Fig. 1B) and FTC clinical stage II specimen shows moderate signal around the thyroid follicles (Fig 1C). Immunohistochemical studies of follicular thyroid carcinoma tissues revealed that PDGFR-α were primarily localized around the follicle. Most of thyroid nodular hyperplasia did not show obvious signals (Fig 1D).Homogeneous positive staining of β-actin is seen in FTC clinical stage I specimen (Fig 1E). There was no specific staining on the tumor tissue with the normal goat serum IgG (Fig 1F).

4.2. Association of PDGFR-α expression with clinicopathological parameters. Thyroid samples from total of 47 patients were used for immunohistochemical analysis of PDGFR-α expression. The correlation between protein expression and the clinicopathological features of the patients is presented in Table I. In estimating staining intensity, we found no statistical correlation between PDGFR-α expression and lymph nodal status(P =0.072), clinical stage(P= 0.451), differentiated status (P= 0.545),metastasis status (P=0.767). In estimating the percentage of cells stained with PDGFR-α antibody, there was no statistical relationship

between PDGFR-α expression and nodal status(P =0.055), differentiated status (P= 0.624). A statistically significant correlation between positive PDGFR-α expression and tumor stage(P=0.01 and P=0.001) was consistently demonstrated in staining intensity and the percentage of staining cells.

4.3. Inhibition of endogenous PDGFR-α production in follicular thyroid carcinoma cell line. We examine the PDGFR-α siRNA inhibit the expression of the PDGFR-α protein in follicular thyroid carcinoma cell line (CGTH W-1). The cell expressing PDGFR-α protein was observed by Immunofluorescence cell staining. At day 4 after transfection with PDGFR-α siRNA once, the expression levels of the PDGFR-α protein shows a moderate reduction (Figure 2F). Furthermore, we performed the double transfection in day0 and day2 to enhance the inhibition effect. Strikingly, the PDGFR-α expression was almost completely repressed.(Figure 2I). However, we examine the expression levels of the PDGFR-α proteins in cell treated with non-silencing siRNA, the siRNA did not affected the PDGFR-α expression (Fig 2C).

V. Discussion

In *in situ* hybridization study of human glioma, PDGFR-α positive cells were found in all malignant grades, although those cells were comparatively denser in tumors of higher malignant levels (grade III and IV)[24]. Expression of the cognate ligand, PDGF-A, increased from low or undetectable levels in less malignant tumors (grade I and II) to high levels in more malignant tumors (grade III and IV)[25,26]. In present study, we have examined the expression levels of PDGFR-α in follicular thyroid carcinoma and non-malignant thyroid nodular hyperplasia (NH) tissues by immunohistochemistry. Tissue sections from follicular thyroid carcinomas showed up-regulated expression levels of PDGFR-α. However, tissue sections of nodular hyperplasias rarely express PDGFR-α. Furthermore, the statistical analyses showed that PDGFR-α expression was increased in 55.5% of thyroid neoplasm and that there was significant correlation between levels of PDGFR-α expression and the tumor stage of clinicopathological parameters. These results show PDGFR-α expression affected thyroid tumor's size or regional invasion. Our data support the notion that up-regulation of PDGFR-α function may be an important step in early carcinogenesis (Part 2).

Recent studies related to RNA interference have shown a process of homology-dependent degradation of cognate mRNA by short-interfering RNA (siRNA) [27-31]. In mammalian cells, it was recently shown that introduction of a small interfering RNA, generally 21-23 nt, could also achieve a similar gene inhibitory effect [32]. To understand whether the abberant expression of PDGFR-α observed from the immunohistochemistry results of follicular thyroid carcinoma could be suppressed by specific siRNAs, thyroid follicular carcinoma cell line (CGTH W-1) possessing the up-regulated PDGFR-α expression were treated with genetic-specific siRNAs against PDGFR-α gene. It have been found that the aberrant expression of PDGFR-α could be suppressed. The thyroid follicular carcinoma cell line (CGTH W-1) and genetic specific siRNAs against PDGFR-α

could be extended to study PDGFR-related signal transduction and understand the role for PDGFR-α in thyroid malignancy (Part 4).

In conclusion, the present study showed that PDGFR-α expression was significantly correlated with the tumor stage of thyroid tumor. The up-regulated PDGFR-α expression might be an indication of early carcinogenesis.

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Part 4

Repression of proliferation in follicular thyroid carcinoma cell line by siRNA –mediated silencing of the PDGFR-α gene

I. Abstract:

Platelet-derived growth factor (PDGF) and its receptor (PDGFR) could transduce the signal of proliferation that may play an important role in thyroid carcinogenesis(in Part 2). Aberrant expressions of PDGF and PDGFR were found in several tumors, including brain tumors, oligoglyomate tumors. Therefore, inhibition of the aberrant signal transduction could have the potential to reduce the possibility of carcinogenesis.

Some of tyrosine kinase inhibitors have shown to block the tyrosine-like receptors and achieve the inhibition of the aberrant signal transduction. Here we designed short interfering RNAs (siRNA) specific for *PDGFR-α* to repress cell proliferation in follicular thyroid carcinoma cell line(CGTH W-1). Real-time quantitative PCR, flow cytometry, immunofluorescence cell staining, and MTT assay results demonstrated that the transfected CGTH W-1 cells reduce the cellular *PDGFR-α* mRNA level, reduce the PDGF α-receptor in the CGTH W-1 cell surface, and repress cell proliferation. While control studies of non-silencing siRNA showed no significant effects in *PDGFR-α* expression and cell proliferation.

Finally, we compared the effects of repressing cell proliferation of CGTH W-1 cells by PDGFR-α siRNA and a tyrosine kinase inhibitor, tyrphostin AG1295. PDGFR-α siRNA required 24 hours more than tyrphostin AG1295 to show significant inhibition of cell proliferation, but the effects last up to 240 hours. The findings indicate that the PDGFR-α siRNA could be a potential tool to suppress aberrant *PDGFR-α* gene expression.

II. Introduction:

Cell proliferation and differentiation are basic events in embryonic development. Development of tumor or embryo may share common ways of sustaining cell proliferation. Therefore, many genes that play important roles in embryogenesis are important in carcinogenesis also. The gene encoding *platelet-derived growth factor α-receptor* (*PDGFR-α*) is one of the genes that are highly active during both tumor and embryonic development. Several studies have found that aberrant expression of PDGFR-α was associated with several tumors(1-4).

The development of clinically useful PDGF antagonists is therefore highly desirable. Several inhibitors inhibiting the function of target proteins belong to the family of PDGF-related signal transduction have been described and have been aimed to identify potent inhibitors that specific for PDGF-related signal transduction but that do not interfere in the effect of other kinases. An interesting and potentially useful approach to inhibit PDGF-related signal transduction is by inhibiting the PDGF receptor kinase (5). Low-molecular-weight compounds, tyrphostins, have been described that efficiently inhibit the activities of tyrosine kinases. Some of these compounds show selectivity for inhibition of PDGF receptor kinases(6-10); however, their precise specificities remain to be determined.

The term RNA interference (RNAi) was devised after the discovery that injection of dsRNA into the nematode *Caenorhabditis elegans* leads to specific silencing of genes highly homologous in sequence to the delivered dsRNA(11). In addition to the nematode, RNAi was also observed in insects(12) , frogs(13), and other animals including mice(14,15) and is likely to also exist in human. RNAi is closely related to the posttranscriptional gene-silencing (PTGS) mechanism of cosuppression in plants and quelling in fungi(16-21), and some components of the RNAi machinery are also necessary for posttranscriptional silenceing by cosuppression(17,19,22). The PTGS mechanism by double-stranded RNA (dsRNA or RNAi) is conserved in a diverse variety of organisms(23) and is mediated by small-interfering RNAs

(siRNAs) that are produced from long dsRNAs of exogenous or endogenous origin by an endonuclease of the ribonuclease-III type, called Dicer. The resulting siRNAs are about 21–23 nucleotides long and are incorporated into a nuclease complex, the RNA-induced silencing complex (RISC), which then targets and cleaves mRNA that is complementary to the siRNA(24).The natural function of RNAi and cosuppression appears to be protection of the genome against invasion by mobile genetic elements such as transposons and viruses, which produce aberrant RNA or dsRNA in the host cell when they become active(25-29).

In mammalian cells , sequence-specific inhibition of gene expression can be achieved by artificially transfecting 19-21 nucleotide small interfering (si)RNAs, which are complementary to the mRNA sequence of a given target gene(30). In the study, the siRNA duplexes for sea pansy (*Renilla reniformis*, RL) and two sequence variants of firefly (*Photinus pyralis*, GL2 and GL3) luciferases were co-transferred with a reporter plasmid in mammalian cells(30). It shows that in NIH/3T3, monkey COS-7 and HeLa S3 cells, GL2, GL3 and RL expression was reduced in response to the cognate siRNAs. Therefore, the analysis of gene function in human cell and the development of gene-specific therapeutics could be further studies by performing RNAi.

To understanding whether the designed PDGFR-α siRNA could specifically reduce the characteristics of carcinogenesis by reducing PDGFR-α expression. We use the CGTH w-1 cell line , which possess an autocrine activation of PDGF-related signal pathway (in Part 2) , to evaluate the inhibition effects of PDGFR-α expression by transfecting specific PDGFR-α siRNAs. Furthermore, we compare the effects of inhibiting cell proliferation between tyrphostin AG 1295 and PDGFR-α siRNA in CGTH W-1 cells. Foregoing studies would offer several implication about PDGFR-α siRNA in suppress the aberrant proliferation and may provide a new tool for suppressing tumor cell proliferation.

III. Materials and methods:

3.1 Cell cultures. CGTH W-1 (derived from metastatic follicular thyroid carcinoma) cell line was obtained from the Division of Endocrinology and Metabolism, Chang Gung Memorial Hospital, Taiwan. Monolayer cultures of CGTH W-1 was grown in RPMI medium 1640 (GIBCO, Invitrogen Corporation, NY) supplemented with 10% fetal calf serum, 2 g/L sodium bicarbonate (SIGMA, Sigma-Aldrich, MO), 1% (v/v) nonessential amino acid (GIBCO), 1 mM sodium pyruvate (GIBCO), 100 U/mL penicillin G sodium, and 100 μg/mL streptomycin(GIBCO).

3.2 Protein tyrosine kinase (PTK) inhibitors: AG1295 and AG9. AG9(Tyrphostin A1: An inactive compound that can be used as a negative control for tyrphostin series. CAS 2826-26-8, $C_{11}H_8N_2O$, M.W. 184.2) and AG1295(Selectively inhibits platelet-derived growth factor (PDGF) receptor kinase (IC_{50}=500nM) and PDGF-dependent DNA synthesis (IC_{50}=2.5μM) in Swiss/3T3 cells. It has no effect on EGF receptor autophosphorylation, shows only weak effect on EGF- or insulin-stimulated DNA synthesis and could be a potent blocker of smooth muscle cell proliferation. CAS 71897-07-9, $C_{16}H_{14}N_2$, M.W. 234.3.) were purchased from Calbiochem (Merck Biosciences, Germany). A stock solution of AG9 and AG1295 (1mM) was prepared with DMSO and diluted with PBS to the required concentrations before each experiment.

3.3 RNA interference. Specific siRNA oligos targeting *PDGFR-α* mRNAs were designed as indicated from Qiagen by using the online siRNA design tool (www.qiagen.com/sirna). Specific and control oligos were synthesized by Qiagen and the oligos were: *PDGFR-α* sence RNA 5'-GGCAC GCCGC UUCCU GAUA TT-3', *PDGFR-α* antisence RNA 5'-UAUC AGGAA GCGGC GUGCC TT-3' ,control siRNA sence RNA :5'-UUCUC CGAAC GUGUC ACGU TT-3' and control siRNA antisence RNA: 5'-ACGU GACAC GUUCG GAGAA

TT-3'(Figure 1). Non-silencing (control) siRNA is 23-nt length dsRNA appearing only 16 bases overlap with the complete genome of *Thermotoga maritimia*. Cells were transfected using Lipofectamine 2000 (INVITROGEN life technologies, Invitrogen Corporation, CA) and samples for quantitative PCR and flow cytometry analysis were taken at the indicated time points.

3.4 Total RNA isolation. Total RNA was obtained by extracting a cell line in Trizol reagent (INVITROGEN life technologies) according to the manufacturer's instructions. Thyroid carcinoma cell lines were homogenized in Trizol solution (1 mL). Homogenates were incubated for 5 minutes at 25℃, and then 0.2 volume of chloroform was added to the homogenates. The inorganic phase was separated by centrifugation at 12,000 g for 20 minutes at 4℃ after vigorous agitation for 5 minutes. RNA was then precipitated in the presence of 0.5 volume of isopropanol. RNA pellets were washed with 70% ice-cold ethanol and then dissolved in RNase-free water. Total RNA concentration was assessed by absorbency at 260 nm using an UV spectrophotometer (Gene Quant II, Pharmacia Biotech, Sweden). RNA quality was confirmed and visualized as 18s and 28s bands in the agarose gel without a smearing pattern.

3.5 Quantitative PCR. Transfected cells grown in 6-well plates were trypsinized and harvested in PBS buffer. Total RNA from harvested cells was extracted by using Trizol reagent (INVITROGEN life technologies) according to the manufacturer's instructions. For reverse transcription, equal amount of total RNA (0.5 μg)were performed in a 25-μl reaction mixture containing 1x reverse transcriptase reaction buffer(Promega, Promega Corporation, WI), 200μM dNTPs, 10ng oligo $(dT)_{15}$ primer, 8mM dithiothreithol, 40 units Rnasin (Promega), and 100 units MMLV reverse transcriptase (Promega). The mixture was incubated at 42℃ for 50 minutes , heated to 70℃ 10 minutes, and then chilled on ice. The GeneAmp

5700 sequence detection system (Applied Biosystems, Applied Biosystems, CA) was used to amplify both target genes and internal control. The reaction master mix was prepared according to the manufacture's protocol to give final concentration of 1x SYBR Green PCR buffer, 3mM $MgCl_2$, dNTP blend(0.2mM dATP, 0.2mM dCTP, 0.2mM dGTP, 0.4mM dUTP), 0.025 units AmpliTaq Gold DNA polymerase, 0.01 units AmpErase uracil-N-glycosylase, and 300nM primers listed below. Complementary DNA synthesized by reverse transcription was added to the master mix. Then, the PCR reagent mix were transferred to thermocycler and PCR profile were performed at 50℃ for 2 min, 95℃ for 10 min, and followed by 40 cycles of amplification at 95℃ for 15 sec, 60℃ for 1 min, using the GeneAmp 5700 sequence detection system.

Oligonucleotide primers

Primer Name	Sequence: (5'-3')
PDGFR-α Forward	5'-TGAAG AAAAC AACAG CGGCC-3'
PDGFR-α Reverse	5'-CGTCA TTCCT AGAGG TACAA AGGCT-3'
β-Actin Forward	5'-ATGGG TCAGA AGGAT TCCTA TGTG-3'
β-Actin Reverse	5'-GCCAG ATTTT CTCCA TGTCG TC-3'

Platelet-derived growth factor receptor-α (*PDGFR-α*), GenBank accession number: M21574; *cytoplasmic β-actin* (*β-Actin*), GenBank accession number:X00351.

Relative expression of *PDGFR-α* transcripts was determined by the following calculation, as described in the Applied Biosystems users bulletin, 'Relative Quantitation of Gene Expression':

Relative expression = $2^{-\triangle\triangle Ct}$

Where $\triangle\triangle Ct = (Ct_{PDGFR-\alpha} - Ct_{\beta-Actin})_{\text{siRNA treatment}} - (Ct_{PDGFR-\alpha} - Ct_{\beta-Actin})_{\text{mock transfection}}$

3.6 Flow cytometry(FCM) analysis. For FCM analysis, CGTH W-1 cells were stained with 1:100 dilution of rabbit polyclonal IgG anti-PDGFRα (C-20; Santa Cruz Biotechnology, Santa Cruz Biotechnology, Inc., CA), then treated with goat anti-rabbit IgG conjugated by fluorescein isothiocyanate (Santa Cruz Biotechnology). FCM analysis was performed with

10,000 cells per condition, using FACSCalibur and CellQuest software (BD Biosciences, Becton-Dickinson, CA).

3.7 Immunofluorescence cell staining. 10^5x CGTH W-1 cells were added to each well containing a glass slide on six-wells plate. The slides were incubated with normal serum of the same species as the second antibody to block nonspecific antibody staining. Rabbit polyclonal IgG anti-PDGFRα (C-20) (Santa Cruz Biotechnology) were applied as primary antibodies at 2 μg/ml in PBS with 1.5% normal blocking serum. Goat anti-rabbit IgG conjugated by fluorescein isothiocyanate (Santa Cruz Biotechnology) were used as secondary antibody diluted to 1 μg/ml in PBS with 1.5% normal blocking serum in dark chamber. Cell nuclei was counter-stained in DAPI. The slides were mounted coverslips with aqueous mounting medium and examined using a laser scanning confocal microscope(Leica TCS SP2-MP System, Leica Microsystems Inc., IL).

3.8 MTT assay. The inhibitory effects of cell proliferation of tyrphostin AG1295 and PDGFR-α siRNA on the CGTH W-1 cells were assessed by a novel MTT-based assay. Briefly, 1x 10^3 or 5x 10^2 CGTH W-1 cells were seeded in each well of 96-well microtiter plates and allowed to attach overnight. The next day (day0) and day 2, the adherent cells were treated with different conditions, including cell alone, mock transfection, non-silencing siRNA, PDGFR-α siRNA, tyrphostin A1 and tyrphostin AG1295. Assays are performed by adding 20μl of the CellTiter 96® Aqueous one solution cell proliferation assay(promega) directly to culture wells, followed by incubation for 1 hour at 37℃. The optical density was determined with a microplate analyzer (Fusion, Packard BioScience Company, CT) at 490nm. Each assay was performed in triplicate.

3.9 Assays for mitogenicity. The proliferation and morphology of CGTH W-1 cells were

assessed by the *in vitro* mitogenic assay. Cells were seeded at $3x10^4$ cells per well of six-well plate, and the adherent cells were treated twice at day0 and day2 with different conditions, including cell alone, mock transfection, non-silencing siRNA, PDGFR-α siRNA, tyrphostin A1 and tyrphostin AG1295. Transfection mixture were left on cells for 6 hours. The monolayers were fixed with 4% formaldehyde and stained with 0.1% crystal violet after 6 days.

3.10 Statistical analysis. The experimental data were analyzed by student's *t* test. The level of statistical significance was set at $p<0.05$.

IV. Results

4.1 PDGFR-α siRNAs reduce the target mRNA in CGTH W-1 cells. A prerequisite for the therapeutic application of siRNAs is that the targeted cells or tissue contain a functional RNAi mechanism to bind to siRNAs and mediate mRNA degradation. Original reports about the successful induction of RNAi in cells of human origin primarily dealt with HeLa cells (30).

To understand whether RNAi mechanism could be observed in thyroid carcinoma cells, CGTH W-1 cells were transfected with PDGFR-α siRNA or control siRNA. We first examined the effect of the PDGFR-α siRNA on the accumulation of cellular *PDGFR-α* mRNA by reverse-transcription, followed by quantitative PCR. The relative *PDGFR-α* mRNA levels were estimated until 120hr after transfection (Fig. 2a). The expression level of *PDGFR-α* mRNA had a sharp drop at 2hr after transfection by the PDGFR-α siRNA. Then, the expression level of the *PDGFR-α* mRNA started to show upward trend from 2hr to 48hr, and restore approximately the normal level as those of cells transfected with non-silencing siRNA form 48h to 120hr (Figure 2a). From the Figure 2b, it can be shown that the cells treated with non-silencing siRNA, tyrphostin A1(AG9) and tyrphostin AG1295 showed similar expression level of *PDGFR-α* mRNA at 2 hr , conversely ,the cells treated with PDGFR-α siRNA show significant reduction. Those results indicated that PDGFR-α siRNA initially inhibited the accumulation of cellular *PDGFR-α* mRNA at the duration of around 2 hr.

4.2 Inhibition of endogenous PDGFR-α production in CGTH W-1 cell line. We examine the PDGFR-α siRNA inhibit the expression of the PDGFR-α protein for the duration of 6 days. The cell number expressing PDGFR-α protein was determined by Flow cytometry (FCM) analysis at different time points after transfection. At day 3 after transfection, the expression levels of the PDGFR-α protein shows a moderate reduction (Figure 3a.). Then, the expression

levels of the PDGFR-α proteins transfected once with PDGFR-α siRNA have moderate reduction by 27% [mean fluorescence intensity(MFI; total fluorescence intensity divided by total counted cell number) shifted from 15.32 to 11.12] as compared with those transfected with non-silencing siRNA(Figure 3b; upper panel). Furthermore, we performed the double transfection to enhance the inhibition effect in day0 and day2. Strikingly, the mean fluorescence intensity of cells transfected with PDGFR-α siRNA was reduce to 58% (MFI was shifted from 13.42 to 5.6) as compared with those transfected with non-silencing siRNA(Figure 3b; lower panel). Finally, we examine the expression levels of the PDGFR-α proteins, including cell alone, mock transfection, non-silencing siRNA, PDGFR-α siRNA, tyrphostin A1 and tyrphostin AG1295 by transfected siRNA twice at day0 and day2. It further shows that the PDGFR-α is significantly reduced expression in PDGFR-α siRNA treatment at day 3 (Figure 3c).

4.3 Immunofluorescence localization of PDGFR-α in CGTH W-1 cells. To observe for silencing of endogenous PDGFR-α proteins appearing on cell membrane, we performed the immunofluorescence cell staining. Silencing was monitored at 72hr after transfection to allow for turnover of the protein of the targeted genes. As shown in Figure 4, the expression of PDGFR-α was specifically reduced by the PDGFR-α siRNA (Fig 4d), but not reduced when non-silencing transfection was performed (Fig 4b). Furthermore, the CGTH W-1 normally show membrance staining of PDGFR-α(Fig 4e) , the CGTH W-1 cells transfected with PDGFR-α siRNA could be observed no membrance staining(Fig 4f).

4.4 CGTH W-1 cells proliferation was suppressed by PDGFR-α siRNA. To study the anti-proliferation effect of PDGFR-α siRNA on the growth of CGTH W-1 cells, CGTH W-1 cells were treated with cell alone ,mock transfection ,non-silencing siRNA, PDGFR-α siRNA, tyrphostin A1 and tyrphostin AG1295. The cell viabilities were quantitated by MTT assay.

When seeding 10^3 cell per well, the proliferation of CGTH W-1 cells were significantly suppressed by transfected with PDGFR-α siRNA in comparison with those by transfected with non-silencing siRNA from day3 to day9 (*p< 0.05;** p< 0.001 ; Figure 5a).On the other hand, the proliferation of CGTH W-1 cells were significantly suppressed by treated with tyrphostin AG1295 in comparison with those by treated with tyrphostin A1 from day2 to day6 (# p< 0.05; ## p< 0.001 ; Figure 5a).

Strikingly, when seeding 500 cells per well, the proliferation of CGTH W-1 cells were significantly suppressed by transfected with PDGFR-α siRNA in comparison with those by transfected with non-silencing siRNA from day2 to day9 (*p< 0.05; ** p< 0.001 ; Figure 5b). On the other, the proliferation of CGTH W-1 cells were significantly suppressed by treated with tyrphostin AG1295 in comparison with those by treated with tyrphostin A1 from day1 to day6 (# p< 0.05; ## p< 0.001 ; Figure 5b).

.In contrast, mock transfection and the tranfection with non-silencing siRNAs little affected the proliferation of CGTH W-1 cells. And, higher ratio of siRNAs to cells could achieve longer inhibition duration and better efficiency, e.g. the 5x 10^2 CGTH W-1 cells transfected with PDGFR-α siRNA were almost not proliferation until day9 (Figure 5b).

When we performed the assays for mitogenicity, we found that the cell monolayer transfected with PDGFR-α siRNA and tyrphostin AG1295 reduce cell appearance (Figure 6). CGTH W-1 cells depleted for PDGFR-α genes show a larger anti-proliferation phenotype than the cells transfected with non-silencing siRNA (Fig.6, day 6). Similar reductions in cell proliferation were also observed in CGTH W-1 cells treated with tyrphostin AG1295. Furthermore, the monolayers of CGTH W-1 cells transfected with PDGFR-α siRNAs show scatter and mottled morphology, those treated with AG1295 showed symmetrical morphology. This indicates that replication of siRNA dupleses may not occur in mammalian cells. It also appears that silencing does not spread to neighboring, non-transfected cells. On the other hand, the cells treated with tyrosine kinase inhibitor (tyrphostin AG1295) would take similar effect

in every cell, so every cell was slowed down the proliferation potential similarly.

V. DISCUSSION

Activation of the PDGFR-α triggers a number of biological responses which in normal cells includes progression through the cell cycle and cell proliferation. When deregulated, the PDGFR-α appear to play an important role in the development of certain types of tumors. For instance, up-regulated expression of the *PDGFR-α* correlated with an elevation of metastatic potential of cell lines derived from lung tumors and ovarian carcinomas(31-33). Furthermore, expression of the PDGFR-α serves as a molecular marker for malignant astrocytomas, and establishment of autocrine loops involving the PDGFR-α and PDGF-AA may be the underlying cause of the hyperproliferation(34-37).

The development of clinically useful PDGF antagonists is therefore highly demanded. An interesting and potentially useful approach to inhibit PDGF signal transduction is to inhibiting the PDGF receptor kinase. Some of tyrphostins show inhibition of PDGF receptor kinases selectively (6, 8-10,38); however, their precise specificities remain to be determined. On the other hand, antisense approaches using single-stranded molecules of either RNA or DNA are relatively straightforward techniques for these purposes. However, questionable specificity or rather low efficacy remained (39-41).

It has recently been discovered that RNAi occurs posttranscriptionally and affects mRNA degradation (42-43). In nature RNAi may play an important biological role in protecting the genome against instability caused by the accumulation of transposons and repetitive sequences(27,29). RNAi in animals may also represent an ancient antiviral response, just as posttranscriptional gene silencing appears to protect plants from viral infection (27,44,45). On the other, RNAi could achieve specific intracellular regulation of gene expression that may provide a new tool for studying gene function in mammalian cells and may afterward be used as gene-specific therapeutics by short 21-23 nucleotides dsRNAs(46-48). It is known that longer RNA in the cytoplasm of mammalian cells can trigger profound physiological reations

that lead to the induction of interferon synthesis(49). In the interferon response, dsRNA>30 bp binds and activates the protein kinase PKR(50) and 2',5'-oligoadenylate synthetase (2',5'-AS)(51).Activation PKR stalls translation by phosphorylation of the translation initiation factors eIF2α,and activated 2',5'-AS causes mRNA degradation by 2',5'-oligoadenylate-actvated ribonuclease L. These responses are intrinsically sequence-nonspecific to the inducing dsRNA.

Among several recently published examples about gene-specific therapeutics by using siRNA, the selective inhibition of the BCR-abl oncoprotein in chronic myelogenous leukemia(CML) cells provides a compelling illustration of the use of RNAi for the assessment of cancer drugs(52). Wilda et al. (52) have recently demonstrated that selective targeting of the BCR-abl oncoprotein with siRNA reduced both the *BCR-abl* transcript and protein levels, which strongly correlated with the induction of apoptotic cell death in leukemic K562 cells. Secondly, Jiang and Milner showed that siRNAs targeting the human papilloma viral (HPV) oncoproteins E6 and E7 in cervical cancer cells led to suppression of cell growth (via *p21* induction) and cause cell death respectively, confirming earlier experiments which suggested that expression of both oncoproteins is required to maintain the malignant phenotype(53). Thirdly, Brummelkamp et al. have also addressed the feasibility of specific gene silencing by using retroviral vectors encoding short hairpin (sh) RNAs targeted to the mutant form of *ras*, the authors specifically and stable inhibited the expression of oncoprotein in human tumor cells(54).Exposure to shRNAs resulted in a loss of anchorage-independent growth in culture and tumorigenicity in nude mice. These results highlight the remarkable specificity that can be achieves by using RNAi and suggest that RNAi can be extended to genetic target validation for candidate drug targets.

In the thyroid follicular carcinoma cell line CGTH W-1, we have found that aberrant expression of *PDGFR-α* can be regulated by PDGFR-α siRNA. PDGFR-α is required for proliferation of CGTH W-1 cells and confer a proliferative advantage when overexpressed.

The PDGFR-α siRNA caused a decrease in *PDGFR-α* gene activity in CGTH W-1 cells. We speculated that PDGFR-α siRNA inhibited the accumulation of *PDGFR-α* mRNAs in transfected cells. In the presence of PDGFR-α siRNA, the newly or existing transcribed *PDGFR-α* mRNAs is degraded, resulting in inhibition of PDGFR-α protein synthesis and gradually reduce the PDGF α-receptor that translocated to the cell surface. Without newly PDGFR-α in the cell surface, further signal transduction and cell proliferation were slowed down or further stopped. Conversely, in the presence of non-silencing siRNAs , newly or existing *PDGFR-α* mRNA were not degraded, the protein expression and cell proliferation were not affected. Despite the RNA degradation is observed at 2 hours and protein expression is repressed at day 3, due to existing PDGF receptor in cell surface, the inhibition of cell proliferation were not observed significantly in single transfection or not significantly different from day0 to 3. Similar phenomenon were observed by Wilda et al., the apoptotic induction by siRNA was delayed by approximately 24h. This delay is probably due to the relatively long half-life of the BCR-abl protein and resulting residual kinase activity, as compared to the direct and rapid enzymatic inhibition of BCR-abl via chemical inhibition (52). The efficacy of siRNAs to silence expression of a given gene can vary, depending on the half-life and abundance of the gene product as well as on the mRNA target sequence and its accessibility (30, 52, 55-58).

As shown here, the short-term existence of PDGFR-α siRNA by transient transfection could inhibited the abberant proliferation of tumor cells. Our data, obtained from MTT analyses, indicate that significant repression of cell proliferation by using RNAi was delayed by approximately 24h as compared to the direct and rapid enzymatic inhibition of PDGFR-α via tyrphostin AG1295. And, higher ratio of siRNAs to cells could achieve longer inhibition duration and better efficiency than lower ratio of siRNAs to cells. This suggests that a larger or longer exposure to siRNAs interfering with PDGFR-α function may suffice to suppress

proliferation, even could completely repress proliferation until day9. Thus, in view of a clinical perspective, a combination treatment of PDGFR-α siRNAs with PTK-related signal pathway inhibitors or a siRNA expression vector which were useful in long-time interference appears to be most promising(59).

Collectively, cancer cells may become growth-dependent on a diverse range of molecular changes. These include oncoproteins that are unique to cancer cells such as the fusion products of chromosomal translocations (e.g. BCR-abl), as well as proteins that are essential to normal cell physiology. Examples of the latter include fatty acid synthase (FASN), whose expression is increased in prostate, breast and ovarian cancers (60). Natural and synthetic FASN inhibitors lead to selective tumor cell apoptosis in experimental models of prostate and breast cancer, with limited toxicity , revealing a hyper-sensitivity in tumor cells that does not appear to be present in normal cells. Similarly, other cancers appear to be susceptible to inhibitors of heat shock protein 90 (61), which is critical to normal protein folding, or inhibitors of the proteosome (62), which plays a central role in normal protein degradation. The observed diversity of "oncogenic dependencies" suggest that significant insights might be expected to come from functional genomic screens that perturb gene transcription or protein function in cancer cells. This is especially true for proteins whose function only becomes essential for survival or proliferation in the context of specific oncogenic phenotypes. Our experiments by using RNAi to perturb *PDGFR-α* expression illustrated that follicular thyroid carcinoma cell line was growth dependence on the change of PDGFR-α expression.

Nevertheless, successful application of siRNA as therapeutical recipe requires an efficient delivery system to target tumor cells *in vivo*. Recently, it was demonstrated that siRNAs can be delivered to organs, predominantly the liver, of postnatal mice by high-pressure tail-vein injection of siRNAs or plasmids that mediate transcription of siRNAs (63,64). Injection of a luciferase reporter gene resulted after 72 h in luciferase expression in organs such as the liver, which could be reduced 75% by co-injection of siRNAs that target

luciferase. Furthermore, co-injection of the vector resulted in 90% reduction of luciferase expression(64). This indicates that siRNAs can be delivered to organs of adult mice and it shows that there is specific siRNA-mediated inhibition of transgenes. The effect was diminished after several days, reflecting the transient nature of this delivery method. Importantly, it was also established that tail-vein injection of siRNAs against EGFP in an EGFP transgenic mouse strain reduced the expression of EGFP in hepatocytes(63). This implies that siRNAs can effectively inhibit the expression of endogenous genes *in vivo*. Although these experiments clearly demonstrate that siRNAs can function *in vivo*, for human disease therapy the design of an effective delivery-system is still required (65).

We report here the identification of PDGFR-α siRNA that can potently inhibit cell proliferation in CGTH W-1 cell line. PDGFR-α siRNA therefore offer one strategy to specifically target *PDGFR-α* gene expression. We show here that inhibition by the potent PDGFR-α siRNA is a result of both sequence-specific interference with cellular *PDGFR-α* mRNA accumulation and reduce the cell surface PDGF receptor. Finally, efficiently inhibiting proliferation appeared in CGTH W-1 cells.

Summary:

Part 1.

1. **Hypothesis:** Diagnostic markers for follicular thyroid carcinoma could be uncovered by comparing expression patterns of cDNA microarrays.
2. **Specific aims:** The profile of differentially expressed oncogenes from human thyroid carcinoma were analysed and confirmed.
3. **Results:**

3.1 18 up-regulated genes and 40 down-regulated genes, which changed their expression levels in metastasis tissue of follicular thyroid cancer, were identified by comparing expression patterns of cDNA microarrays.

3.2 41 up-regulated and 38 down-regulated genes, which changed their expression in thyroid follicular carcinoma cell line(CGTH W-1), were identified as compared with those in benign tissues of thyroid nodular hyperplasia.

3.3 35 up-regulated and 22 down-regulated genes, which changed their expression in papillary thyroid carcinoma cell line(CGTH W-3), were identified as compared with those in benign tissues of thyroid nodular hyperplasia.

3.4 Candidate genes (*CDC-RPK, c-fos, Gelatinase A, IGFBP*), which have showed the differential expression between primary thyroid carcinoma and its metastasis tissue, showed the same expression pattern by gene-specific RT-PCR as observed using the cDNA expression arrays.

3.5 Five interesting genes (*GRB2 isoform, c-myc MM-1, SOD1, fau, GAPDH*), which have showed distinct expression in follicular thyroid carcinoma cell line by using cDNA microarray, were reconfirmed by using RT-PCR. Only the gene expressions of *c-myc MM-1, SOD1* showed the similar expression pattern as observed using the cDNA expression arrays.

4. **Conclusion:** The cDNA array technique is an effective tool for monitoring the overall profile of gene expression in thyroid follicular carcinoma cells. We applied RT-PCR method to confirm mRNA expression of candidate genes. However, determining how relevant the expression difference of a particular gene is to the underlying malignance process remains to be solved (Part 2). The study of thyroid malignance could help to determine whether candidate genes play an important role in thyroid carcinogenesis.

Part 2.

1. **Hypothesis:** After analysis of differentially gene expression by a cDNA microarray technique, we found that mRNA of PDGF-A and PDGFR-α were highly expressed in thyroid carcinomas cells but not in nodular hyperplasia cells(Part 1). We proposed that over-expression of PDGF-A and PDGFR-α cause autocrine activation, then accelerating cell proliferation.
2. **Specific aims:** The over-expressions of PDGF-A and PDGFR-α proteins were primarily observed in cell models of thyroid carcinoma cell lines (CGTH W-1 and CGTH W-3 cell). Then, we survey the PDGFR-α activation in terms of phosphorylation. Finally, we observed whether the activity of PDGFR-α tyrosine kinase was necessary for CGTH W-1 and CGTH W-3 cell proliferations.
3. **Results:**

3.1 Among candidate genes examined from expression patterns of cDNA microarray, PDGF-A mRNA exhibited 2- and 2.7-fold over-expression in CGTH W-1 and CGTH W-3, respectively, and PDGFR-α mRNA exhibited 13- and 137.5-fold over-expressed in CGTH W-1 and CGTH W-3, respectively. The mRNA expression of PDGF-A and PDGFR-α in nodular hyperplasia were not found.

3.2 PDGF-B mRNA did not show any obvious expression among nodular hyperplasia, CGTH W-1 cell line and CGTH W-3 cell line. The mRNA of PDGF β-receptor shows

no obvious expression in both nodular hyperplasia tissues and CGTH W-1 cell line, and only slight expression in CGTH W-3 cell line.

3.3 The gene expressions of PDGF-A and PDGFR-α amplified by gene-specific quantitative PCR displayed the same tendency as observed by the cDNA expression arrays.

3.4 The protein level of PDGF-A was detected by antibodies against an epitope of PDGF-A (135-211 amino acids). The result shows that a band of approximately 26 kDa was detected in CGTH W-1 and CGTH W-3, while it was not detected in nodular hyperplasia.

3.5 The protein level of PDGFR-α was detected by antibodies against an epitope of PDGFR-α (carboxyl terminus). The polyclonal antibody against the PDGFR-α shows that marked bands around at 180, 156, 130, 90 and 52 kDa in total protein lysate of CGTH W-1 and CGTH W-3.

3.6 PDGFR-α phosphorylation was detected by a western blotting with phosphor-specific antibodies. The results showed that several bands (170, 156, 120, 90 and 52KDa) were observed in CGTH W-1 and CGTH W-3 cell lines by using antibodies against p-PDGFR-α (Tyr720), while no obvious band was observed in same cell lines by using antibodies against p-PDGFR-α (Tyr754).

3.7 When different concentrations of tyrphostin AG1295 were added to the thyroid carcinoma cell lines, the result showed a dose-dependent inhibition on cell proliferation. After the 7-day, the average rate of inhibiting cell proliferation with adding different concentrations of tyrphostin AG1295 (0, 0.25, 0.5, 1, and 2.5 μM) were 0%, 7%, 42%, 51%, and 82% in CGTH W-1 cells and 0%, 13%, 29%, 36%, and 51% in CGTH W-3 cells.

4. Conclusion: Both PDGF-A and PDGFR-α were over-expressed in the follicular thyroid carcinoma (CGTH W-1) and papillary thyroid carcinoma (CGTH W-3) cell lines. In thyroid

cells, those aberrant expressions could develop an abnormal signal transduction route by the PDGF-related autocrine activation and consequently enhanced cell proliferation. The over-expression of PDGF-A and PDGFR-α genes might be an indication of carcinogenesis. However, it must be solved whether the aberrant gene expression could be observed in clinical thyroid tissues or only in specific cell lines (Part 3).

Part 3.

1. **Hypothesis:** We proposed that PDGFR-α expression was distinguishable between benign thyroid nodule and malignant thyroid carcinoma.
2. **Specific aims:** We would like to identify whether the aberrant expression of PDGFR-α was related to the advanced malignant thyroid cells. Then, we observed the correlation between PDGFR-α expression and the clinicopathological features.
3. **Results:**

   3.1 Immunohistochemical studies of follicular thyroid carcinoma tissues revealed that PDGFR-α were primarily localized around the follicle. However, most of thyroid nodular hyperplasia did not show obvious PDGFR-α expression. Positive immuno-staining for PDGFR-α was 20/36 (55.5%) cases of follicular thyroid carcinomas.

   3.2 Estimating staining intensity in follicular thyroid carcinoma, we found no statistical correlation between PDGFR-α expression and lymph nodal status(P =0.072), clinical stage(P= 0.451), differentiated status (P= 0.545),metastasis status (P=0.767).

   3.3 Estimating the percentage of cells stained with PDGFR-α antibody, there was no statistical relationship between PDGFR-α expression and nodal status(P =0.055), differentiated status (P= 0.624).

   3.4 A statistically significant correlation between positive PDGFR-α expression and tumor stage(P=0.01 and P=0.001) was consistently demonstrated in staining intensity and the

percentage of staining cells.

4. **Conclusion:** These results showed that PDGFR-α expression was significantly correlated with the tumor stage of thyroid tumor and might affect thyroid tumor's size or regional invasion. The up-regulated PDGFR-α expression might be an indication of early carcinogenesis.

Part 4.

1. **Hypothesis:** In part 2, we found the positive signal transduction route in follicular thyroid carcinoma cells. We proposed that negative regulation method, RNA interference, could restore hyper-proliferated cells to normal status.
2. **Specific aims:** We would like to identify whether inhibition of the aberrant PDGFR-α expression could have the potential to dismiss the possibility of carcinogenesis?
3. **Results:**

3.1 The expression level of *PDGFR-α* mRNA had a sharp drop at 2hr after PDGFR-α transfection. Then, the expression level of the *PDGFR-α* mRNA started to show upward trend from 2hr to 48hr, and restore approximately the normal level as those of cells transfected with non-silencing siRNA form 48h to 120hr.

3.2 CGTH W-1 cells treated with non-silencing siRNA, tyrphostin A1(AG9) and tyrphostin AG1295 showed similar expression level of *PDGFR-α* mRNA at 2 hr , conversely ,the cells treated with PDGFR-α siRNA show significant reduction. These results indicated that PDGFR-α siRNA initially inhibited the accumulation of cellular *PDGFR-α* mRNA at the duration of around 2 hr.

3.3 The expression levels of the PDGFR-α proteins transfected once with PDGFR-α siRNA have moderate reduction by 27% [shifted from 15.32 (MFI) to 11.12] as compared with those transfected with non-silencing siRNA.

3.4 The mean fluorescence intensity of cells transfected with PDGFR-α siRNA twice in

day0 and day2 was reduce to 58% (MFI was shifted from 13.42 to 5.6) as compared with those transfected with non-silencing siRNA.

3.5 The expression levels of the PDGFR-α proteins estimated in different conditions, including cell alone, mock transfection, non-silencing siRNA, PDGFR-α siRNA, tyrphostin A1 and tyrphostin AG1295 show significantly reduced expression in PDGFR-α siRNA treatment at day 3.

3.6 In immunofluorescence staining, the CGTH W-1 cell normally show membrance staining of PDGFR-α, while the cells transfected with PDGFR-α siRNA are repressed the membrance staining.

3.7 When seeding 10^3 cells per well, the proliferation of CGTH W-1 cells were significantly suppressed from day3 to day9 by transfected with PDGFR-α siRNA in comparison with those by transfected with non-silencing siRNA. On the other hand, the proliferation were significantly suppressed from day2 to day6 by treated with tyrphostin AG1295 in comparison with those by treated with tyrphostin A1.

3.8. When seeding 500 cells per well (higher ratio of siRNAs to cells), the proliferation of CGTH W-1 cells were significantly suppressed from day2 to day9 by transfected with PDGFR-α siRNA in comparison with those by transfected with non-silencing siRNA. On the other, the proliferation were significantly suppressed from day1 to day6 by treated with tyrphostin AG1295 in comparison with those by treated with tyrphostin A1. Higher ratio of siRNAs to cells could achieve longer inhibition duration and better efficiency, e.g. the 5x 10^2 CGTH W-1 cells transfected with PDGFR-α siRNA were almost not proliferation until day9.

3.9 The monolayers of CGTH W-1 cells transfected with PDGFR-α siRNAs show scatter and mottled morphology, those treated with AG1295 showed symmetrical morphology. This indicates that replication of siRNA dupleses may not occur in mammalian cells. It also appears that silencing does not spread to neighboring, non-transfected cells. On the

other hand, the cells treated with tyrosine kinase inhibitor (tyrphostin AG1295) would take similar effect in every cell, so every cell was slowed down the proliferation potential similarly.

4. **Conclusion:** We show here that inhibition by the potent PDGFR-α siRNA is a result of both sequence-specific interference with cellular *PDGFR-α* mRNA accumulation and reduce the cell surface PDGF receptor. Finally, efficiently inhibiting proliferation appeared in CGTH W-1 cells.

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Tables

Part of introduction

Table 1. TNM Clinical Classification of Thyroid Cancer

T - primary tumor

Tx- can not be assessed

T0 - no evidence of primary tumor
T1 - single tumor under 2 cm, confined to the gland
T2 - single tumor. >2 and < 4 cm, limited to the thyroid
T3 - tumor > 4 cm, limited to thyroid, or minimal extension to strap muscle or perithyroidal soft tissue
T4a-Tumor extends beyond thyroid capsule into subcutaneous soft tissues, larynx, trachea, esophagus , or RCN
T4b-Tumor invades prevertebral fascia, mediastinal vessels, or encases carotid

N - regional lymph nodes
Nx- can not be assessed
N0 - no palpable nodes
N1 - Regional lymph node metastases
N1a-Metastasis in Level VI (pretracheal and paratracheal, including prelaryngeal and delphian lymph nodes
N1b-Metastasis to other unilateral, bilateral or contralateral cervical or mediastinal nodes

M - distant metastases
Mx- can not be assessed
M0 - no evidence of distant metastases
M1 - distant metastases present

Stage I- T1 N0 M0, = or > 45 yr
Stage II- M1 < 45 yr, or T2 N0 M0 = or> 45 yr
Stage III- T3 N0 M0 = or > 45 yr, T1-3 N1a M0 = or> 45 yr
Stage IV- T1-3 N1b M0 = or> 45 yr, T4a N0-1 M0 = or > 45 yr
T4b Nx M0 = or > 45 yr, Tx Nx M1 = or > 45 yr

Table 2. Comparison of Two Clinical Staging Systems

Clinical Stage	Comparable TNM Classification
I: Intrathyroidal	T0, T1, T2, N0, M0
II: Cervical adenopathy	T0-T2, N0, N1a, N1b, M0
III: Locally invasive disease	T3, T4a, T4b M0
IV: Distant metastases	M1

Part 1.

TABLE 1. Listing of genes identified using the technique of cDNA arrays in metastatic and follicular thyroid cancer

GenBank accession no.	Name of protein/gene	Ratio
Genes upregulated in metastatic thyroid tissues		
L29216	CLK-2	3.33
X66363	Serine/threonine-protein kinase PCTAIRE-1	2.96
L33264	CDC2-related protein kinase (PISSLRE)	7.78
U40343	CDK inhibitor p19 (INK4d)	21.95
M31159	Growth hormone-dependent insulin-like growth factor-binding protein	2.93
L35253	P38 mitogen activated protein(MAP) kinase	9.44
J00124	Cytokeratin 14	2.39
M26326	Cytokeratin 18	2.53
X03212	Cytokeratin 7	5.22
M77198	Rac protein kinase beta	6.02
K00650	c-fos	11.14
S40706	GADD153 growth arrest and DNA-damage-inducible	5.94
X65923	FAU	5.34
M38690	CD9	2.46
U72661	ninjurin	27.9
L20471	extracellular matrix metalloproteinase inducer emmprin	62.75
X87838	Beta-catenin	16.23
M23410	Desmoplakin III	7.41
Genes downregulated in metastatic thyroid tissues		
X51688	Cyclin A	0.50
S72008	CDC 10 protein homolog	0.50
M15796	PCNA;cyclin	0.19
L29511	GRB2 isoform	0.28

L08246	**Induced myeloid leukemia cell differentiation protein MCL-1**	**0.50**
U13699	**Caspase-1**	**0.42**
K03214	**Proto-oncogene c-src1 tyrosine kinase domain**	**0.09**
M19722	**Fgr proto-oncogene**	**0.37**
J04088	**DNA topoisomerase II alpha**	**0.06**
U07418	**DNA mismatch repair protein hmlh 1**	**0.09**
D21235	**UV excision repair protein RAD23**	**0.38**
M76125	**AXL tyrosine kinase receptor**	**0.45**
X00588	**epidermal growth factor receptor**	**0.26**
M37722	**basic FGF receptor 1 precursor**	**0.30**
M21574	**PDGF-alpha receptor**	**0.04**
M21616	**PDGF-beta receptor**	**0.26**
J04599	**byglycan**	**0.11**
X55525	**collagen type I**	**0.12**
X15879	**collagen type VI alpha-1**	**0.07**
M34570	**collagen type VI alpha-2**	**0.08**
X52022	**collagen type VI alpha-3**	**0.001**
X57527	**collagen type VIII alpha-1**	**0.44**
M92642	**collagen type XVI alpha-1**	**0.10**
U43901	**laminin 3-kDa receptor**	**0.18**
X78565	**tenascin-C**	**0.13**
J03040	**basement membrane protein BM-40**	**0.49**
X02761	**fibronectin**	**0.11**
X05231	**collagenase-1**	**0.02**
J03210	**gelatinase A**	**0.19**
D26512	**MMP-14**	**0.27**
Z30183	**Mitogen-inducible gene5(mig-5)**	**0.001**
X04429	**endothelial plasminogen activator inhibitor-1 precursor(PAI-1)**	**0.17**
X95282	**Pho8 protein**	**0.09**
M64595	**RAS-related C3 botulinum toxin substrate**	**0.01**
M77349	**BIGH3**	**0.19**
U36223	**Fibroblast growth factor-8(FGF-8)**	**0.17**

M57399	**Nerve growth factor HBNF-1**	**0.24**
M25639	**MIF**	**0.44**
U41745	**PDGF associated protein**	**0.46**
X06374	**Platelet-derived growth factor A chain**	**0.22**

Table 2. A list of differentially expressed genes identified by using a cDNA array technique in the ratio of follicular thyroid carcinoma cell line to benign tissue (F/B ratio), together with the ratio of papillary thyroid carcinoma cell line to benign tissue (P/B ratio).

GenBank Accession no.	*Name of protein/gene*	*F/B Ratio*	*P/B Ratio*
A	**Cell cycle & growth regulators**		
X05360	Cell division control protein 2 homolog	0.36 ↓	0.68
X66365	Cell division protein kinase 6 (CDK6); PLSTIRE	0.45 ↓	1.08
M34065	CDC25C;M-phase inducer phosphatase 3	2.64 ↑	1.93
L25676	Cell division protein kinase 9 (CDK9); PITALRE	0.15 ↓	0.43 ↓
L33264	Cdc2-related protein kinase PISSLRE	0.26 ↓	0.54
X59798;M64349	G1/S-specific cyclin D1(CCND1); bcl-1 oncogene	0.43 ↓	1.54
M92287	G1/S-specific cyclin D3(CCND3)	0.50 ↓	1.17
U11791;U12685	Cyclin H(CCNH); MO 15-associated protein	0.16 ↓	0.68
U09579;L25610	WAF1	0.23 ↓	0.84
S72008	CDC10 protein homolog	2.55 ↑	3.40 ↑
U00001	CDC27HS protein	0.30 ↓	1.22
U63131	CDC37 homolog	0.45 ↓	0.96
X60188	Extracellular signal-regulated kinase 1(ERK1)	0.38 ↓	1.08
L35253;L35263	Mitogen-activated protein kinase p38 (MAP kinase p38)	3.80 ↑	3.22 ↑
X85134	RBQ-3	2.89 ↑	3.01 ↑
AF001954	P33ING1	0.39 ↓	0.61
L29511	Growth factor receptor-bound protein 2 (GRB2) isoform	0.41 ↓	1.45
X03484	*c-raf proto-oncogene*	0.47 ↓	0.92
M29039	Jun-B	2.65 ↑	3.73 ↑
D89667	c-myc binding protein MM-1	0.27 ↓	1.09
M26326	Type I cytoskeletal 18 keratin (K18)	0.18 ↓	0.22 ↓
M34225	Type II cytoskeletal 18 keratin (KRT8)	0.10 ↓	2.10 ↑
B	**Apoptosis, oncogenes & tumor suppressors**		
L08246	Induced myeloid leukemia cell differentiation Protein MCL-1	0.32 ↓	1.35
S83171;Z35491	BCL-2 binding athanogene-1(BAG-1)	0.23 ↓	0.55

L41690	Tumor necrosis factor receptor 1-associated death domain protein (TRADD)	0.20 ↓	0.80
AF016268	Cytotoxic TRAIL receptor 2 (TRICK2A)	2.84 ↑	2.40 ↑
M35543;M57298	CDC42 homolog	0.42 ↓	0.74
D17517	Tyrosine-protein kinase receptor tyro3 precursor;rse;sky;dtk	2.27 ↑	2.98 ↑
C	**Cell fate & development regulators**		
J04088	DNA topoisomerase II alpha (TOP2A)	10.55 ↑	64.12 ↑
M60974	DNA-damage-inducible transcript 1 (DDIT1)	2.46 ↑	11.39 ↑
M87339	Activator 1 37-kDa subunit; replication factor C 37-kDa subunit(RFC37)	2.53 ↑	76.12 ↑
L07541	Replication factor C 38-kDa subunit (RFC38)	2.76 ↑	5.23 ↑
M87338	Replication factor C 40-kDa subunit (RFC40)	2.63 ↑	32.60 ↑
K00065;X02317	Cytosolic superoxide dismutase 1(SOD1)	0.36 ↓	1.41
U94354	Lunatic fringe	3.13 ↑	15.71 ↑
X91940	Wnt-8B	0.46 ↓	1.06
U46461	Segment polarity protein; disheveled homolog 1 (DSH homolog 1)	0.46 ↓	1.78
L38518	Sonic hedgehog (SHH)	2.77 ↑	8.61 ↑
M76125	Tyrosine-protein kinase receptor UFO precursor; axl oncogene	2.71 ↑	68.15 ↑
X65923	fau	0.49 ↓	1.23
M29366;M34309	ERBB-3 receptor protein-tyrosine kinase precursor	2.40 ↑	4.60 ↑
M34641	Fibroblast growth factor receptor 1 precursor (FGFR1)	2.75 ↑	10.20 ↑
M21574	Platelet-derived growth factor receptor alpha subunit (PDGFRA)	13.00 ↑	137.48 ↑
U12140	Brain-derived neurotrophic factor (BDNF)/NT-3 growth factors receptor precursor	0.25 ↓	0.95
M32315;M55994	Tumor necrosis factor binding protein 2 (TBP2)	2.76 ↑	1.34
D	**Cell adhesion, motility & invasion**		
J04599	Bone/cartilage proteoglycan 1 precursor	2.63 ↑	2.36 ↑
X55525;J03464	Procollagen 1 alpha 2 subunit precursor (COL1A2)	2.85 ↑	2.77 ↑
X15879	Collagen 6 alpha 1 subunit (COL6A1)	2.21 ↑	4.26 ↑

X52022	Collagen 6 alpha 3 subunit (COL6A3)	12.17 ↑	6.40 ↑
J03040	Secreted protein acidic and rich in cysteine precursor (SPARC)	2.15 ↑	1.64
X02761	Fibronectin precursor (FN)	7.51 ↑	4.77 ↑
U12431+U29943	ELAV-like neuronal protein 1(HEL-N1) +HEL-N2	2.91 ↑	2.75 ↑
M59911	Integrin alpha 3(ITGA3)	0.50 ↓	0.89
L12002	Intergrin alpha 4 precursor (ITGA4)	2.46 ↑	1.76
M38690	CD9 antigen	0.17 ↓	0.93
X51521	Ezrin;villin 2(VIL2)	0.20 ↓	0.60
Z18951;S49856	Caveolin-1	3.39 ↑	7.22 ↑
X60957;S89716	Tyrosine kinase receptor tie-1 precursor	0.16 ↓	0.01 ↓
E	**Invasion regulators, cell-cell interactions**		
X05232	Matrix metalloproteinase 3 (MMP3)	2.67 ↑	4.49 ↑
D26512;X83535	Matrix metalloproteinase 14 (MMP14)	2.61 ↑	6.54 ↑
Z30183	Metalloproteinase inhibitor 3 precursor; tissue inhibitor of metalloproteinases 3(TIMP3)	4.79 ↑	2.05 ↑
L20471	Basigin precursor (BSG)	0.21 ↓	1.28
X17620	NDP kinase A; tumor metastatic process-associated protein	2.30 ↑	3.17 ↑
X95282	Rho-related GTP-binding protein RhoE	3.69 ↑	5.26 ↑
X87838;Z19054	Beta catenin(CTNNB)	0.26 ↓	0.64
M23410;Z68228	Junction plakoglobin (JUP); desmoplakin III (DP)	0.24 ↓	0.29 ↓
M59371;M36395	Epithelial cell kinase (ECK)	4.73 ↑	3.65 ↑
F	**Growth factors, cytokines & chemokines**		
M32977;M27281	Vascular endothelial growth factor precursor (VEGF)	0.10 ↓	0.78
M77349	BIGH3	3.62 ↑	2.68 ↑
D49493	Growth differentiation factor 10 (GDF 10)	2.85 ↑	0.61
M60828	Fibroblast growth factor 7 (FGF7)	0.06 ↓	0.01 ↓
X52946	Heparin-binding growth factor 8 (HBGF-8)	2.90 ↑	3.14 ↑
A25270	Interferon gamma antagonist (INF-gamma antagonist)	0.50 ↓	1.43
M27544+M37484	Insulin-like growth factor IA precursor (IGF1A);IGFBP1	0.40 ↓	1.27
M57627	Interleukin-10 precursor (IL-10)	0.50 ↓	0.34 ↓

A03911	Glia-derived neurite-promoting factor (GDNPF)	2.70 ↑	1.46
X06374	Platelet-derived growth factor A subunit precursor (PDGFA;PDGF1)	2.00 ↑	2.68 ↑
L09753	CD30 ligand(CD30L);CD153 antigen	2.13 ↑	0.76
L07414	Tumor necrosis factor (TNF)-related activation protein (TRAP)	2.97 ↑	1.00

Part 3.

Table 1. Correlation between PDGFR-α expression and clinicopathological parameters in 47 patients with thyroid neoplasm.

	Number		Intensity of PDGFR-α			Percentage of cells stained with PDGFR-α antibody		
	n=47	%	Negative	Positive(%)	P	Negative	Positive(%)	P
Sex								
Male	12	25.5						
Female	35	74.5						
Age								
<45	14	29.8						
≥45	33	70.2						
Tumor stage					0.01			0.001
T0*	11	19.1	11	0		10	1(2.1)	
T1	9	21.3	5	4(8.5)		6	3(6.4)	
T2	13	27.7	6	7(14.9)		5	8(17.0)	
T3	6	12.8	1	5(10.6)		0	6(12.8)	
T4	8	19.1	4	4(8.5)		1	7(14.9)	
Lymph node					0.072			0.055
N0	31	66.0	21	10(21.3)		19	12(25.5)	
N1	11	23.4	4	7(14.9)		3	8(17.0)	
Nx[#]	5	10.6						
Clinical stage (n=36)					0.451			0.03
Stage I	7	19.4	4	3(8.3)		5	2(5.6)	
Stage II	9	25.0	5	4(11.1)		5	4(11.1)	
Stage III	9	25.0	2	7(19.4)		1	8(22.2)	
Stage IV	11	30.6	5	6(16.7)		2	9(25.0)	
Metastasis (n=36)					0.767			0.047
Negative	20	55.5	8	12(33.3)		8	12(33.3)	
Positive	16	44.4	8	3(22.2)		2	14(38.8)	

***No evidence of primary tumor ; [#] can not be assessed**